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L10 ANSWER 2 OF 14 CA COPYRIGHT 2003 ACS
AN 136:128460 CA
TI Pharmacological induction of fetal hemoglobin in sickle cell disease and .beta.-thalassemia
AU Atweh, George F.; Loukopoulos, Dimitris
SO Seminars in Hematology (2001), 38(4), 367-373
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TI Induction of hemoglobin F synthesis in patients with .beta. thalassemia
AU Ley, Timothy J.; Nienhuis, Arthur W.
SO Annual Review of Medicine (1985), 36, 485-98

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TI 5-Azacytidine acts directly on both erythroid precursors and progenitors to increase production of fetal hemoglobin
AU Humphries, R. Keith; Dover, George; Young, Neal S.; Moore, Jeffrey G.; Charache, Samuel; Ley, Timothy; Nienhuis, Arthur W.
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5-Azacytidin Acts Dir ctly n B th Erythroid Pr ur ors and Prog nit rs t Increas Pr ducti n f F tal H m gl bin

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Abstract

The effect of 5-azacytidine on erythroid precursors and progenitors was studied in nine patients with sickle cell anemia or severe thalassemia. Each patient received the drug intravenously for 5 or 7 d. 5-Azacytidine caused a four- to sixfold increase in γ-messenger RNA concentration in bone marrow cells of eight of the nine patients and decreased the methylation frequency of a specific cytosine residue in the γ-globin gene promoter in all nine patients. Within 2 d of the start of drug treatment there was a rise in the percentage of reticulocytes containing fetal hemoglobin (HbF; F-reticulocytes) without a significant change in the total number of reticulocytes, which suggested that there was a direct action of 5-azacytidine on erythroid precursors. Late erythroid progenitors (CFU-E), present in bone marrow after 2 d of drug administration, formed colonies containing an increased amount of HbF as compared with control colonies. Moreover, the number of CFU-E derived colonies was not decreased at these early times, which suggested that there was a direct action of 5-azacytidine on erythroid progenitors in the absence of cytotoxicity. Exposure of normal bone marrow cells in tissue culture to 5-azacytidine for 24 h reproduced both of these effects as judged during subsequent colony formation. The combined direct effects of 5-azacytidine on both the erythroid precursor and progenitor compartments resulted in an increase in HbF synthesis that was sustained for 2–3 wk. Toxicity to bone marrow as reflected by cytoreduction was evident after treatment in some patients but was not accompanied by an increase in HbF production. A correlation was found between the effects of 5-azacytidine on bone marrow, as assessed by in vitro measurements, and the hematological response of the individual patients to drug treatment.

Introduction

The severe hemoglobinopathies or β-thalassemias reflect alterati ns in the structure or function of the β-globin gene, respectively. The onset of these genetic disorders of hemoglobin occur after the perinatal switch from fetal hemoglobin (HbF)[1] ($\alpha_2\gamma_2$) to HbA ($\alpha_2\beta_2$) production as β-globin synthesis replaces γ-globin synthesis. Reactivation of the glo:n genes with replacement of the defective β^S chain, or as compensation for deficient β-synthesis, could be effective treatment for sickle cell anemia or the β-thalassemias.

Normal humans produce a low level of HbF after the first year of life. Usually ≤1% of the total hemoglobin is HbF and it is restricted to a small proportion of the total erythrocytes called F-cells (1). The formation of F-cells appears to be modulated during both erythroid precursor and progenitor development. There is asynchronous synthesis of HbF and HbA during erythroblast maturation; fetal hemoglobin is produced predominantly in the early pro- and basophilic erythroblasts (2–4). Erythroid progenitors from adult bone marrow form colonies of erythroblasts containing substantially more HbF than is found in vivo (5). The erythroid colonies produced in culture from progenitors of a single individual produce highly variable amounts of HbF (6–9). This evidence that modulation of HbF synthesis also occurs during progenitor differentiation, and the clinical observation that HbF synthesis increases transiently after bone marrow transplantation or recovery from erythroblastopenia (10, 11) suggests that HbF synthesis might be reactivated with appropriate stimuli in patients with mutations in the β-globin genes.

Several molecular correlates of hemoglobin switching have been defined. Regulation appears to occur at the level of gene transcription (12). There are changes in chromatin structure as reflected by the disappearance of nuclease-sensitive sites immediately upstream from the γ-globin genes when these genes are switched off during development (13). The cytosine residues in this region are relatively undermethylated during γ-gene expression in the fetus but become fully methylated in adult bone marrow, where the γ-globin genes are repressed (14). The latter observation prompted the use of 5-azacytidine, a cytosine analogue that inhibits the enzymatic methylation of newly synthesized DNA, in an effort to activate the globin genes, first in experimental animals (15) and later in humans (16–18).

Administration of 5-azacytidine led to a nearly complete switch from adult to fetal hemoglobin synthesis in baboons and a substantial increase in HbF synthesis in humans. This increase in HbF production was accompanied by improved production of erythrocytes in thalassemic patients (16) and by a reduction in sickling propensity of the erythrocytes in patients with sickle cell anemia (17). Reduced methylation upstream from the γ-gene accompanied the increase in γ-gl bin gene expression (16–18). Alth ugh the reduction in methylati n was a global ffect in that many cytosine residues showed decreased methylati n frequency, only the γ-globin gene among several genes assayed showed a significant increase in expression.

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1. *Abbreviations used in this paper:* BFU-E, primitive erythroid progenitor(s), burst-forming unit(s); CFU-E, late erythroid progenitor(s); FCS, fetal calf serum; HbF, fetal hemoglobin; IMDM, Iscove's modification of Dulbecco's medium; kb, kilobases.

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This unexplained specificity, together with the known cytotoxic effects of the drug, suggested a mechanism of action for 5-azacytidine independent of its direct effects on DNA methylation. Recent experimental studies in baboons have shown that 5-azacytidine is cytotoxic to progenitors, leading to the hypothesis that the increase in HbF production occurs in part due to regeneration from earlier erythroid progenitors with a higher likelihood of forming erythroblasts that make HbF (19). That hydroxyurea, a cytotoxic drug with no known direct effects on DNA methylation, increases HbF synthesis in anemic monkeys has been interpreted as supporting this type of mechanism (20).

Our studies on the effect of 5-azacytidine in humans have included a detailed examination of the effect of this drug on erythroid progenitors and precursors. In this paper we describe the cellular and molecular data obtained from the study of nine patients, and these results are correlated with the clinical response of each individual patient. The results imply a complex mechanism of action but suggest that the response to 5-azacytidine is at least in part due to a direct noncytotoxic effect of the drug on erythroid precursors and progenitors.

Methods

Characteristics of patients studied

Nine patients were studied in detail. Each was over the age of 21 yr and had either sickle cell anemia or severe (homozygous) β-thalassemia (Table I). Detailed clinical descriptions of patients 1–5 have been previously published (16, 17). Patients 6–9 had a severe sickle syndrome with incapacitating chronic pain, intermittent acute pain crisis, and a significant transfusional iron burden with secondary hemochromatosis. Limited data are also presented on three additional patients with sickle cell anemia treated at Johns Hopkins Hospital, Baltimore, MD; their clinical features have been (18, 21), or will be, described in detail elsewhere.

Table I. Characteristics of Patients Studied

No.	Patient	Age	Diagnosis	Treatment regimen*
1	J.S.	58	Sickle cell anemia	A
2	M.G.	42	Sickle cell anemia	A
3	T.A.	22	Beta thalassemia	A
4	R.R.	22	Beta thalassemia	A
5	C.H.	42	Beta thalassemia	B
6	W.J.	31	Sickle cell anemia	B
7	A.L.	39	Sickle cell anemia	B
8	J.I.	31	Sickle cell anemia	B
9	W.J.J.	40	Sickle cell anemia	1. B‡
				2. A
10	J.P.	34	Sickle cell anemia	C
11	D.P.	28	Sickle cell anemia	C
12	W.T.	36	Sickle cell anemia	C

The treatment course for patients 1–4 (17), an earlier used course for patient 5 (16), and the clinical response of patient 10 (18) have been reported in detail previously. For patients 1–4 and 6–9, the treatment course described was the first the patient received. Patient 5 had received another course of therapy 6 mo earlier.

* A, 2 mg/kg per d given by continuous intravenous infusion for 7 d. B, 1.5 mg/kg per d given over 8 h for 5 d. C, 2 mg/kg per d given for 3 d by bolus injection in divided doses every 8 h.

‡ This patient received two treatment courses separated by several months.

The protocol for drug administration was approved by the National Heart, Lung, and Blood Institute Review Board and informed consent was obtained. Two treatment regimens were used. Patients treated with regimen A received 5-azacytidine (2 mg/kg per d) by continuous infusion for 7 d as described previously (16, 17). Patients treated with regimen B received a single, daily intravenous infusion of 5-azacytidine (1.5 mg/kg per d) over 8 h for 5 successive days. Three patients treated at Johns Hopkins Hospital received 2 mg/kg per d given in divided doses intravenously at 8-h intervals (regimen C). Several patients experienced mild to moderate nausea and vomiting which was controlled with antiemetics, but no other significant side effects were observed.

After informed consent was obtained, hematologically normal volunteers donated bone marrow for various in vitro studies. A protocol for obtaining bone marrow from normal individuals was approved by the National Heart, Lung, and Blood Institute Review Board.

DNA and RNA analysis

RNA and DNA were recovered from bone marrow cells after lysis in guanadinium HCl/sarcosyl as previously described (22). RNA was analyzed with S_1 mapping (23) or primer extension techniques (24). For S_1 mapping, total bone marrow RNA was annealed to uniformly radiolabeled DNA probes prepared on single stranded templates of M13mp7-γ or β-globin gene recombinants as previously described (25). Hybridization and S_1 digestion conditions followed published protocols (23). After S_1 nuclease digestion, samples were precipitated with ethanol, heat denatured, and analyzed on 8% polyacrylamide sequencing gels with subsequent autoradiography (26). α-globin mRNA was analyzed by primer extension by use of an oligonucleotide primer (P-L Biochemicals, Inc., Milwaukee, WI) corresponding to nucleotides 97 through 115 3′ to the cap site. The primer was 5′-labeled by the use of polynucleotide kinase and purified by published techniques (27). The radiolabeled probe and RNA were coprecipitated and lyophilized. After primer extension with reverse transcriptase, extended products were resolved on 8% polyacrylamide sequencing gels. Densitometric analysis of radiographs provided a quantitative estimate of relative mRNA concentrations.

The frequency of cytosine methylation near the γ-globin genes was ascertained by restriction enzyme analysis. Total bone marrow DNA was first digested to completion with the enzyme Eco RI and then further digested with a fivefold excess of the enzyme HpaII (14, 16, 17). The latter enzyme cleaves only if the internal cytosine of the DNA sequence recognized by HpaII [CCGG] is unmethylated. Restriction enzymes were obtained from Bethesda Research Labortories (Gaithersburg, MD) and used according to the manufacturer's recommendations. Completeness of digestion was determined by standard techniques. After double digestion, samples were electrophoresed on 1% agarose vertical slab gels and DNA was transferred to nitrocellulose filters by the technique of Southern (28). Filters were baked, prehybridized, and washed as previously described (29). Filters were then autoradiographed at −70°C by the use of intensifying screens.

Analysis of HbF

Total HbF. HbF in peripheral blood was determined from the percentage in hemolysates as measured by alkaline denaturation (30) and reference to total hemoglobin levels.

F-reticulocytes. Reticulocytes containing HbF were defined by microscopic immunoprecipitate reactions by use of rabbit anti-human HbF antibody as described in detail elsewhere (31). The absolute concentration of F-reticulocytes was calculated by determination of the percentage of total reticulocytes in peripheral blood smears, stained with new methylene blue, and by determination of the red cell count with a Coulter counter (Coulter Electronics Inc., Hialeah, FL).

HbF content of individual erythroid cells. Microscopic immunoprecipitate reactions were also used to determine the percentage of nucleated erythroid cells containing HbF and the amount of HbF in individual erythroid cells present in erythroid colonies grown in vitro

(7, 31). In brief, maximally hemoglobinized colonies derived from mature or primitive erythroid progenitors were plucked, washed, and pooled as described below. Cells were washed three times in buffered saline and dispersed into single cells and mixed with agarose, new methylene blue, and antibody to either human HbF or adult hemoglobin. The agarose suspension was placed n glass slides, the cells were lysed with 0.2% Triton X-100, and the percentage of cells with HbF-anti-HbF or HbA-anti-HbA pericellular immunoprecipitate reactions was enumerated. The amount of HbF or HbA in individual nucleated cells was quantitated microdensitometrically.

Culture of erythroid progenitors

Bone marrow cells aspirated from the posterior iliac crest were drawn into Iscove's modification of Dulbecco's medium (IMDM; Flow Laboratories, McLean, Virginia) with 50 U/ml of preservative-free heparin (O'Neal. Jones & Feldman, St. Louis, MO). Mononuclear cells were separated by Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) sedimentation according to the manufacturer's directions followed by two washes in IMDM-2% heat inactivated (56°C, 30 min) fetal calf serum (FCS; Flow Laboratories) at a final concentration of 1×10^5 cells/ml. Erythroid progenitors culture were assayed in medium containing 0.8% methylcellulose (Dow Chemical Company, Midland, Michigan), 30% FCS, 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri); 100 U/ml penicillin/streptomycin (MA Bioproducts, Walkerville, Maryland); 2.5 U/ml of erythropoietin (Connaught Laboratories, Willowdale, Ontario), and 10% phytohemagglutinin (Gibco Laboratories, Grand Island, New York) stimulated leukocyte-conditioned media. Cultures were incubated at 37°C in 95% humidity and 5% CO_2. Erythroid progenitor types were assayed by their distinctive colony-forming characteristics as follows: colonies derived from the late erythroid progenitor (CFU-E) were counted on day 8 and characterized by a single cluster of well-hemoglobinized cells; colonies derived from more primitive erythroid progenitors, burst-forming units (BFU-E), were scored on day 18 and characterized as consisting of three or more clusters of well-hemoglobinized cells (32). In our laboratory the number of colonies in cultures of 10^5 normal bone marrow mononuclear cells is as follows: CFU-E derived, 93±11, and BFU-E derived, 104±11 (mean±SEM, $n = 22$).

For determination of HbF content of individual erythroid cells contained in erythroid colonies, mature well-hemoglobinized colonies were plucked on day 8 (CFU-E derived colonies) or day 18 (BFU-E derived colonies) under microscopic visualization by use of a tapered Pasteur pipette. Analysis of BFU-E derived colonies was restricted to those derived from the most primitive progenitors by plucking of only those colonies consisting of >16 clusters (32). Approximately 50 CFU-E derived colonies or 5–10 primitive BFU-E derived colonies were pooled, gently resuspended, and washed in buffered saline, and HbF was measured as described above.

In vitro treatment with 5-azacytidine. Bone marrow mononuclear cells from normal volunteers, separated by Percoll density sedimentation, were added at a final concentration of 2×10^6 cells/ml to liquid cultures containing 5-azacytidine at concentrations from 0.1 to 10 μg/ml in 30% FCS, 1% BSA, 10^{-4} M β-mercaptoethanol without erythropoietin, or 10% phytohemagglutinin-stimulated leukocyte-conditioned medium in IMDM at 37°C. To maintain 5-azacytidine concentrations, additional 5-azacytidine was added 8 and 16 h after initiation of incubation. After 24 h, cells were washed and replated in standard methylcellulose culture (see above) at 1×10^5 cells/ml in the absence of 5-azacytidine. CFU-E derived colonies were scored 8 d later. The HbF content of individual erythroblasts in these colonies was determined by radial immunodiffusion as described above.

Results

Molecular effects of 5-azacytidine

Messenger RNA. The relative content of γ-messenger RNA (mRNA) in bone marrow erythroblasts was measured by S_1 nuclease analyses. To compare individual bone marrow samples, γ-mRNA levels were normalized to α- or β-mRNA content as measured by primer extensi n or S_1 nuclease analysis, respectively. The data shown in Fig. 1 *A* were derived from an analyses of bone marrow samples obtained from patient 7 on day 0, 2, or 7. Relative to the pretreatment day 0 value, γ-mRNA content was increased 1.4-fold on day 2 and 5.9-fold on day 7 as determined by demisometric analyses of the radioautographs. An increase in γ-mRNA content was documented on day 2 in three patients studied and on day 7 in all patients (data not shown), with the exception of patient 9. For this patient, during the first course of treatment, there was a small increase in γ-mRNA conten: (1.5×) on day 7 as compared with control obtained on day 0, but there was no detectable increase in γ-mRNA content during the second course on day 3 as compared with the day 0 bone marrow RNA sample (Fig. 1 *B*).

Methylation. A decrease in the frequency of methylation at several sites within the β-like gene cluster was documented by restriction endonuclease analysis and Southern blotting as previously described (14, 16). A representative analysis of the methylation site immediately 5′ to the G_γ- and A_γ-genes for bone marrow DNA samples from patient 9 is shown in Fig. 2. Double digestion of DNA with the enzymes Eco RI and HpaII will yield a 1.5-kilobase fragment if DNA is unmethylated at the HpaII site. A decrease in the frequency of methylation as compared with that of the control DNA samples was apparent upon visual inspection and scanning of the radioautographs of Southern blots in the day 7 bone marrow sample from the first course and in the day 3 bone marrow sample from the second course. Similar data were obtained on analyses of DNA from all patients (references 16 and 17, and data not shown).

Effects of 5-azacytidine on erythroid cells

Reticulocytes. Reticulocytes containing HbF (F-reticulocytes) were enumerated to provide an index of the early effect of 5-azacytidine on erythroid cells. By 7 d, five patients studied in detail showed a 3- to 10-fold increase in percentage of F-reticulocytes (Fig. 3). For 3 of 5 patients, a striking increase was evident after only 24 h of treatment; the other two showed a more gradual increase. This increment in the percentage f F-reticulocytes occurred without a significant change in the absolute reticulocyte count (mean pretreatment, 3.3×10^5 vs. mean day 2–4, 3.1×10^5).

Peripheral blood nucleated red cells. Six of the nine patients reported in detail here showed an increase in absolute nucleated erythrocyte count in the peripheral blood after treatment with 5-azacytidine as previously described (18) (Table II). The maximal increment occurred on days 7–10 and ranged from 3- to 13-fold. All six patients who showed an increase in this parameter have sickle cell anemia. In contrast, the three patients whose nucleated erythrocyte count decreased or did not change have homozygous β-thalassemia.

Erythroid progenitors. The next series of experiments was designed to evaluate the effects of 5-azacytidine treatment on primitive and mature erythroid progenitors. Serial bone marrow samples were obtained bef re, during, and after the course of 5-azacytidine and cultured in the absence of 5-azacytidine. The data obtained by study of patients treated by continuous infusion (Fig. 4, *A* and *B*) are plotted separately from those

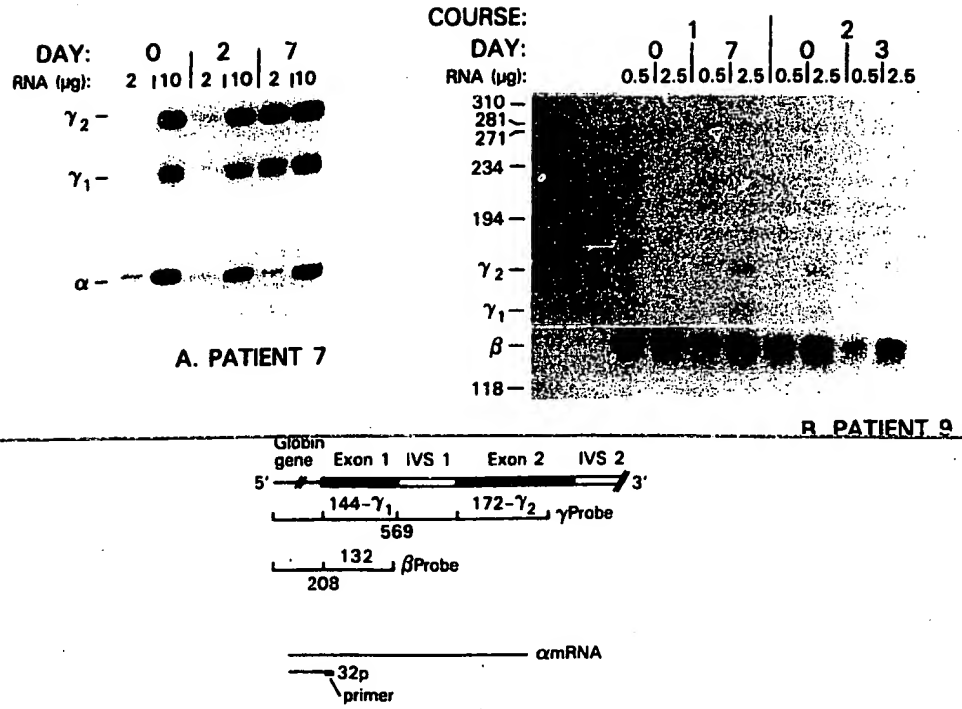

Figure 1. Analysis of γ-globin mRNA in bone marrow obtained from patients receiving 5-azacytidine. (*A*) Response of patient 7. γ-globin mRNA was assessed by S_1 nuclease mapping with a uniformly labeled γ-globin gene-specific probe. As a control, α-mRNA was assessed by primer extension by use of an α-globin gene-specific probe. The origin of the probes used is described in Methods and is depicted at the bottom of the figure with reference to a prototypical globin gene. The expected fragments protected from S_1 nuclease digestion or resulting from primer extension are indicated above the lines representing the probes. The numbers below the line indicate the total length of the probe. The day of treatment on which bone marrow was obtained and the amount and total RNA analyzed are shown above each lane. Each RNA sample was analyzed in replicate by hybridization to either the γ-specific probe or the α-specific probe followed by S_1 nuclease or primer extension, respectively. Radiolabeled products were resolved on separate sequencing gels with subsequent autoradiography. The results obtained for the α-specific probe are shown directly below the corresponding lanes of the γ-specific probe. (*B*) Response of patient 9. For this patient, total bone marrow RNA was simultaneously assessed for γ- and β-globin mRNA by annealing RNA samples to a mixture of the γ-specific probe as described in *A* and a β-specific probe spanning the 5' end of the gene as depicted in the lower part of the figure. The day on which the bone marrow was taken and the amount of RNA used in the hybridization are indicated at the top of each lane. After S_1 nuclease treatment, samples were analyzed on sequencing gels with subsequent autoradiography.

obtained by study of patients who received the bolus infusions (Fig. 4, *C* and *D*). Of the six patients whose marrow was assayed on day 2 of treatment, only one showed a modest decrease of 30% in CFU-E as compared with the pretreatment value, whereas the remainder showed stable or even increased values (0–65%). Two additional patients, for whom no data was available from day 2, were found to have a striking increase in colony numbers in bone marrow samples cultured on day 7. Cytotoxicity to the progenitor compartment (as reflected by a decrease in CFU-E derived colonies) was evident by day 7 or later for five patients. The variablility in colony formation among six normal individuals (each studied 2 or 3 times) expressed as an average standard deviation of the means was 13.3 with a range of 42–162 CFU-E derived colonies/10^5 bone marrow mononuclear cells. Thus, we believe that the changes observed during and after treatment are not due to random variation between samples.

Detailed analyses of HbF content in erythroblasts derived from erythroid progenitors in vitro were obtained for three patients who showed an increase in CFU-E numbers on either day 2 (patients 7 and 8) or day 7 (patient 5) of the treatment course. Well-hemoglobinized colonies derived from mature progenitors were identified and plucked from cultures on the eighth day in vitro and pooled, and the HbF content of individual erythroblasts was determined by radial immunodiffusion. These data are presented in Fig. 5 as pl ts of the cumulative frequency of erythroblasts containing different amounts of HbF. By day 2 of treatment, tw patients (Fig. 5, *A* and *B*), and by day 7 of treatment, all three patients studied, showed an increase in HbF content in CFU-E derived erythroblasts, as reflected by a shift in the cumulative frequency plot to the right and by an increase in the mean HbF content per erythroblast. These data show that in vivo exposure of bone marrow to 5-azacytidine for 2 or 7 d yields CFU-E progenitors whose phenotype is demonstrably altered; erythroblasts generated in vitro from these progenitors contain increased amounts of HbF in the absence of further exposure to 5-azacytidine.

In four of the seven patients, the number of colonies derived from BFU-E remained constant or increased during or after the 5-azacytidine treatment course, whereas in three patients a decrease in BFU-E derived colonies was evident in one or more bone marrow samples subsequent to treatment (Fig. 4). One of the two patients (number 7) whose CFU-E derived erythroblasts from day 2 marrow contained increased amounts of HbF also had an increase in HbF in BFU-E derived colonies from the day 2 sample (mean day 0, 12.94 pg vs. mean day 2, 25.6 pg). In others the base-line HbF in BFU-E colonies was high, preventing meaningful analyses.

Manipulation of progenitors with 5-azacytidine in vitro

The above analyses of erythroid progenitors suggest that short term exposure in vivo to 5-azacytidine altered CFU-E, leading them to f rm erythroid c l nies containing increased amounts of HbF. This alteration occurred in several patients without a decrease in CFU-E numbers or other overt evidence of cytotoxicity. T gain further insight into the mechanisms involved,

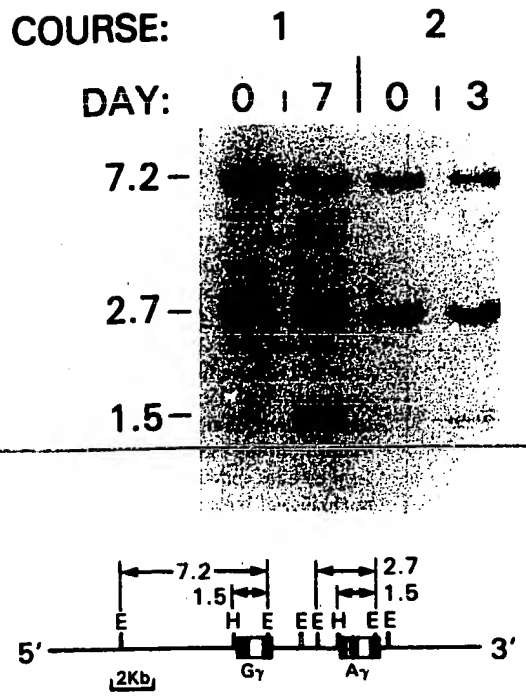

Figure 2. Southern blot analysis of DNA methylation in the region of the human γ-globin genes for patient 9, treatment courses 1 and 2. Each lane represents an analysis of total bone marrow DNA (80–90% erythroid cells) obtained on the indicated day of treatment. Samples were digested to completion with Eco RI and HpaII as described in Methods. Expected fragment sizes are depicted on the schematic of the γ-globin gene region shown at the bottom of the figure. Flanking sequence DNA is indicated by a line, coding sequences are indicated by black boxes, and intervening sequences are indicated by open boxes. The sizes of fragments expected to hybridize with a nick-translated γ-probe derived from the large intervening sequences of the G_γ-gene are indicated. The presence of a 1.5-kb band indicates an absence of cytosine methylation at the HpaII site shown. An additional 5.2-kb band seen in some lanes represents cross hybridization of the probe to a 5.2-kb Eco RI fragment containing the 5' end of the human β-globin gene. No HpaII sites are found within this fragment.

we examined the effects of exposing progenitors to 5-azacytidine in vitro.

Fig. 6 shows the effect on CFU-E derived colony numbers f exposing bone marrow cells from normal volunteers to

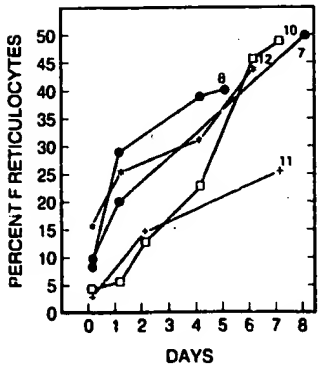

Figure 3. F-reticulocyte response to 5-azacytidine. The percentage of F-reticulocytes in peripheral blood is indicated as a function of days after starting of 5-azacytidine administration. Solid circles with corresponding patient number indicate values from patients with sickle cell anemia treated at the National Institutes of Health (see Table I). The remaining three responses are those for sickle cell anemia patients treated at the Johns Hopkins Hospital.

*Table II. Effect of 5-Azacytidine on Nucleated Erythrocyte Counts**

No.	Patient	Base line	Days 1–5	Days 7–10
1	J.S.	258	0	1327 (5.1)‡
2	M.G.	372	45	1470 (4.0)
3	T.A.§	502	363	155 (0.3)
4	R.R.§	440	684	437 (1.0)
5	C.H.§	1230	319	177 (0.14)
6	W.A.J.	155	239	1964 (12.7)
7	A.L.	700	811	1920 (2.7)
8	J.I.	160	300	2120 (13.3)
9	W.J.J.	1. 75	38	704 (9.4)
		2. 32	50	320 (10)

* Values shown are nucleated erythrocytes per cubic millimeter of peripheral blood and were calculated from inspection of peripheral blood smears and reference to total erythrocyte counts.
‡ -Fold change days 7–10 as compared with base line.
§ Patients have beta-thalassemia. All others have sickle cell anemia.

different 5-azacytidine concentrations in liquid culture for 24 h. After liquid culture the cells were washed and plated in methylcellulose in the absence of 5-azacytidine and CFU-E derived colonies scored. Significant reductions in colony numbers were not observed until 5-azacytidine concentrations above 1.0 μg/ml were used. At low concentrations an increase in colony number was apparent. The number of BFU-E derived colonies remained constant until 10 μg/ml (data not shown).

Based on these results, bone marrow cells from two normal volunteers were exposed to nontoxic concentrations of 5-azacytidine for 24 h before plating in methylcellulose culture in the absence of 5-azacytidine was done. In colonies derived from the CFU-E of both individuals, there was an increase in the percentage of HbF containing erythroblasts at doses of 5-azacytidine that did not significantly reduce colony number (Table III). At higher concentrations a statistically significant increase in HbF per erythroblast was also observed for the colonies derived from bone marrow cells of both individuals, although in one case (experiment 2) this was accompanied by a substantial reduction in colony number (Table III).

Correlation of the effect of 5-azacytidine on erythropoiesis with the clinical response in individual patients

Good responders. 5-Azacytidine caused a progressive increase in HbF concentration and an increase in the total hemoglobin concentration in several patients. Such a response has previously been described in patients 1–5 (13, 14); the clinical course of two additional patients (8 and 7) is shown in Fig. 7. These two patients who have sickle cell anemia had a prompt increase in the absolute F-reticulocyte count, an increase in the nucleated erythrocyte count, and a documented increase in mRNA content in bone marrow erythroblasts during and after 5-azacytidine administration. The concentration of bone marrow erythroid progenitors that formed colonies remained stable (e.g., patient 8, Fig. 7) or increased (e.g., patient 7, Fig. 7) by day 2 of treatment and there was a documented increase in HbF in these colonies as compared with control colonies (Fig. 5).

Certain patients who xhibit the clinical response described above n netheless show a decrease in colony numbers by day

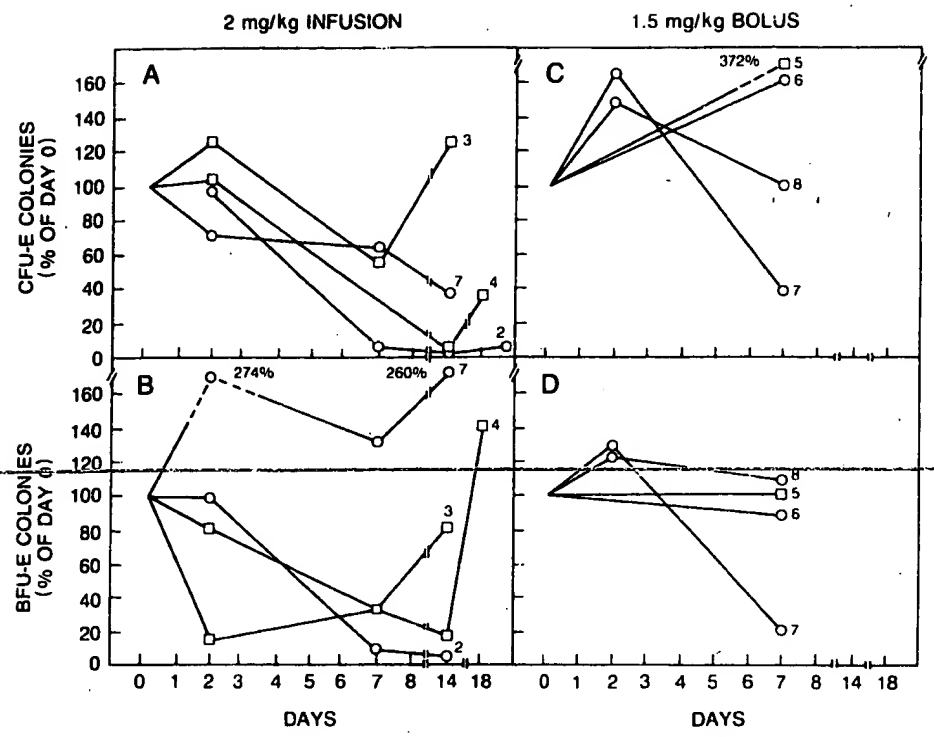

Figure 4. Changes in bone marrow erythroid progenitor number in patients receiving 5-azacytidine. Responses are identified by the patient number as presented in Table I. *A* and *C* depict colonies derived from CFU-E and *B* and *D* show BFU-E as a function of time after the initiation of 5-azacytidine treatment. Colony numbers were determined from the mean value in replicate 1-ml cultures containing 1×10^5 mononuclear bone marrow cells; values are expressed as a percentage of the pretreatment level (day 0). The data in *A* and *B* were derived from study of patients who received 7 d of continuous infusion (regimen A); data in *C* and *D* were derived from study of patients who received a daily 8-h infusion for 5 d (regimen B).

7 (patients 2 and 7). In one case this decrease in colony numbers was found to correlate with a fall in reticulocyte count as described for patient 2 previously. Relevant data are not available for patient 7.

Moderate responders. Patient 6 represents a different pattern of response. This patient exhibited a striking increase in absolute F-reticulocytes that was accompanied by a progressive rise in the HbF concentration in blood to approximately six times higher than the pretreatment level but not by a rise in total hemoglobin (Fig. 8). The increase in HbF concentration in blood between days 5 and 15 did not result in a significant increase in total hemoglobin concentration. Apparently the magnitude of the increase in HbF was compromised by a rebound absolute reticulocytosis and a fall in the absolute F-reticulocyte count between days 9 and 14. These in vivo results correlated with the in vitro culture data. As compared with the day 0 sample, the number of mature progenitors (CFU-E) that formed colonies had increased twofold in the day 7 sample, consistent with rebound absolute reticulocytosis. The erythroblasts derived from these progenitors in vitro showed an increase in HbF content as compared with control (Fig. 9).

Nonresponder. Finally, one patient (number 9) had no significant increase in HbF concentration after treatment; this patient's hemoglobin concentration fell after treatment regimen B (Fig. 10). He was treated a second time with regimen A, which was previously found to be effective in patients 1–5. Serial measurement of F-reticulocytes were made and the behavior of progenitors before and after initiation of 5-azacytidine was determined. Only a very modest increase in HbF concentration was found in peripheral blood; this increase occurred several days after the treatment course was completed (Fig. 10). Toxicity to the bone marrow was reflected by a striking fall in absolute reticulocyte count. The absolute F-reticulocyte count remained relatively constant, however, resulting in a moderate increase in the percentage of F-reticulocytes. Accordingly, this period of relative reticulocytopenia but relative increase in F-reticulocytes was followed by a modest increase in HbF concentration. Restoration of reticulocyte numbers to near base-line values between days 23 and 27 was not accompanied by an increase in the number of F-reticulocytes. Consistent with these findings, the HbF content of erythroblasts present in CFU-E derived colonies were identical in cultures of bone marrow obtained on day 0 and day 3; thus his progenitor cells were apparently not altered upon exposure to 5-azacytidine in vivo (Fig. 9 *B*).

Of interest in this nonresponder, the base-line HbF content per erythroblast in his colonies was higher than that found in the colony erythroblasts of any other patient. Even though there was no clinical or in vitro evidence of an increase in HbF synthesis, a decrease in the frequency of methylation in the region of the γ-globin genes was documented during both treatment periods (Fig. 2).

Discussion

Our studies have documented the time course of the effect of 5-azacytidine on the erythroid precursor and progenitor cell compartments. A direct effect on the erythroid precursor compartment is suggested by an early rise in F-reticulocytes and by an early increase in bone marrow cell γ-mRNA content. The earliest effect of 5-azacytidine on the progenitor compartment is an increase in the amount of HbF in the CFU-E without overt cytotoxicity to this compartment as compared with control colonies. Finally, we have found considerable heterogeneity in the clinical response to 5-azacytidine as reflected by HbF and hemoglobin concentrations. In general there was a correlation between the in vitro measurements of the effect of 5-azacytidine on progenitors and precursors and the clinical response of individual patients.

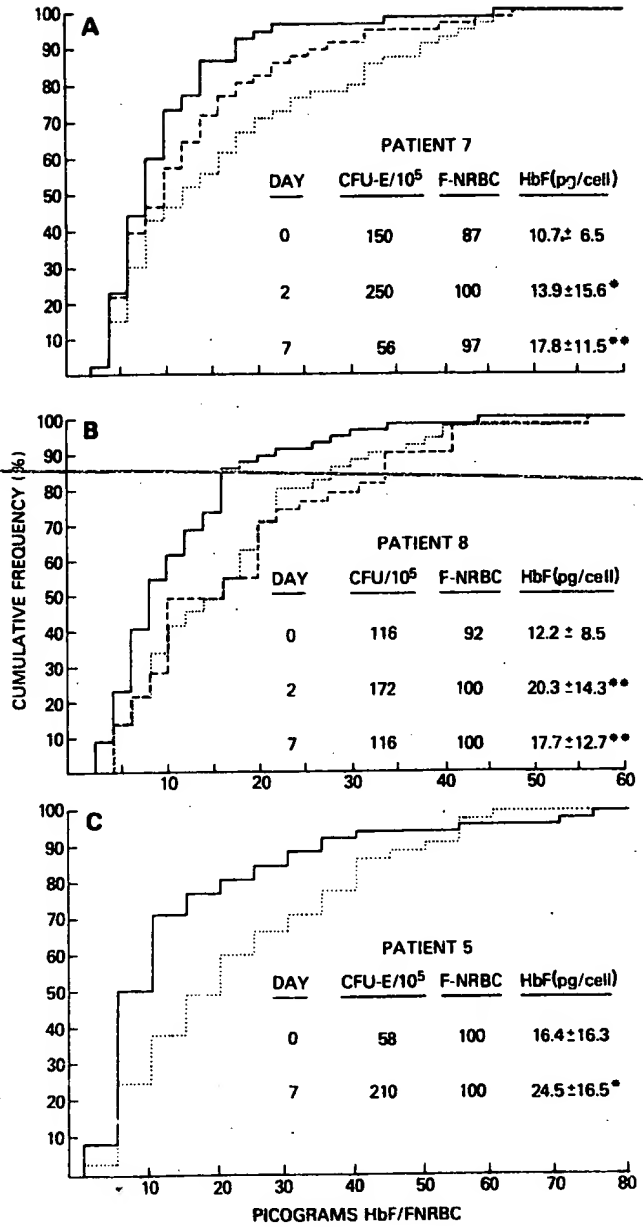

Figure 5. HbF content of nucleated erythrocytes generated in vitro by CFU-E from patients treated with 5-azacytidine. Well-hemoglobinized colonies derived from CFU-E were identified and plucked from cultures of bone marrow cells on the eighth day in vitro; colonies were pooled and resuspended, and HbF content of individual erythroblasts was determined by radioimmunodiffusion. Data are presented as a cumulative frequency plot of cells containing up to a given amount of HbF. Note that colony erythroblasts contain 40–60 pg of total hemoglobin. *A*, *B*, and *C* depict response of patients 7, 8, and 5, respectively (see Table I). Responses are shown for bone marrow taken before initiation of 5-azacytidine administration (day 0, ——) and at day 2 (– – –) and/or day 7 (· · · · ·) after the initiation of therapy. F-NRBC, percentage of nucleated erythrocytes that contain HbF. *, $P < 0.05$. **, $P < 0.01$.

The careful study of Torrealba-de Ron et al. (19) revealed toxic effects of 5-azacytidine on erythroid progenitors of baboons (*Papio cynocephalus*) during the induction of HbF synthesis. 5 d of drug treatment were given at a dose of 5 mg/kg; 1 d later (corresponding to day 6 of our treatment schedules) the numbers of erythroid colonies (derived from CFU-E) and erythroid clusters (derived from pro-erythroblasts) were significantly reduced as compared with pretreatment values. The number of burst colonies was unaffected by 5-azacytidine treatment. 6 d later (corresponding to day 12 of our treatment schedules) the number of clusters and colonies that formed in bone marrow cultures was increased over those observed in

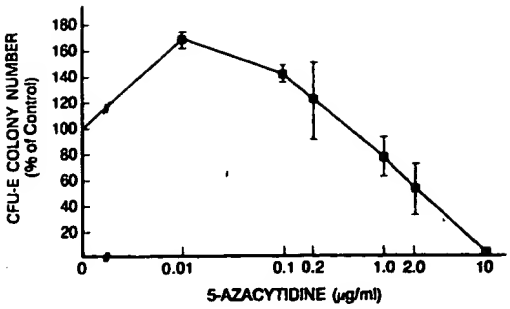

Figure 6. Toxicity profile for CFU-E after exposure to 5-azacytidine in vitro. Bone marrow cells from normal volunteers were placed in liquid culture containing 5-azacytidine at concentrations ranging from 0.1 to 10 μg/ml as described in Methods. Five separate experiments were performed. To maintain the specified concentrations, additional 5-azacytidine was added 8 and 16 h after initiation of incubation (this drug is very unstable after reconstitution). After 24 h, cells were washed and replated in methylcellulose at 1×10^5 cells/ml in the absence of 5-azacytidine. CFU-E derived colonies were scored 8 d later. Results from individual experiments were expressed as a percentage of values obtained in the absence of 5-azacytidine, and the mean and standard error of the mean, obtained upon pooling results from three to five experiments per point were plotted.

*Table III. Effect of 5-Azacytidine on Normal Marrow Progenitors In Vitro**

5-Aza	CFU-E	%FNRBC	HbF/FNRBC
μg/ml	$\times 10^5$ cells		pg
Experiment 1			
0	92	24.4±3.2	5.0±2.2
0.2	140	38.8±2.3‡	5.8±2.1
1.0	74	36.8±1.4‡	11.3±10.1‡
Experiment 2			
0	46	61.3±4.6	6.6±3.3
0.05	56	59.3±2.3	9.0±7.0
0.2	72	68.0±2.0§	8.4±9.6
1.0	17	83.3±1.2‡	9.8±6.3‡

* Bone marrow mononuclear cells from two normal adults (experiments 1 and 2) were incubated in liquid culture in vitro with 5-azacytidine (5-Aza), and then washed and plated in methylcellulose culture without 5-azacytidine. CFU-E derived colonies were counted and HbF analyzed in cells from pools of plucked colonies by the radial immunodiffusion assay. Values shown are means±SD. For determination of percentage of nucleated erythroblasts containing HbF (FNRBC), 250 cells were counted; the HbF/FNRBC was determined for 50–100 cells.
‡ $P = 0.01$.
§ $P = 0.05$.

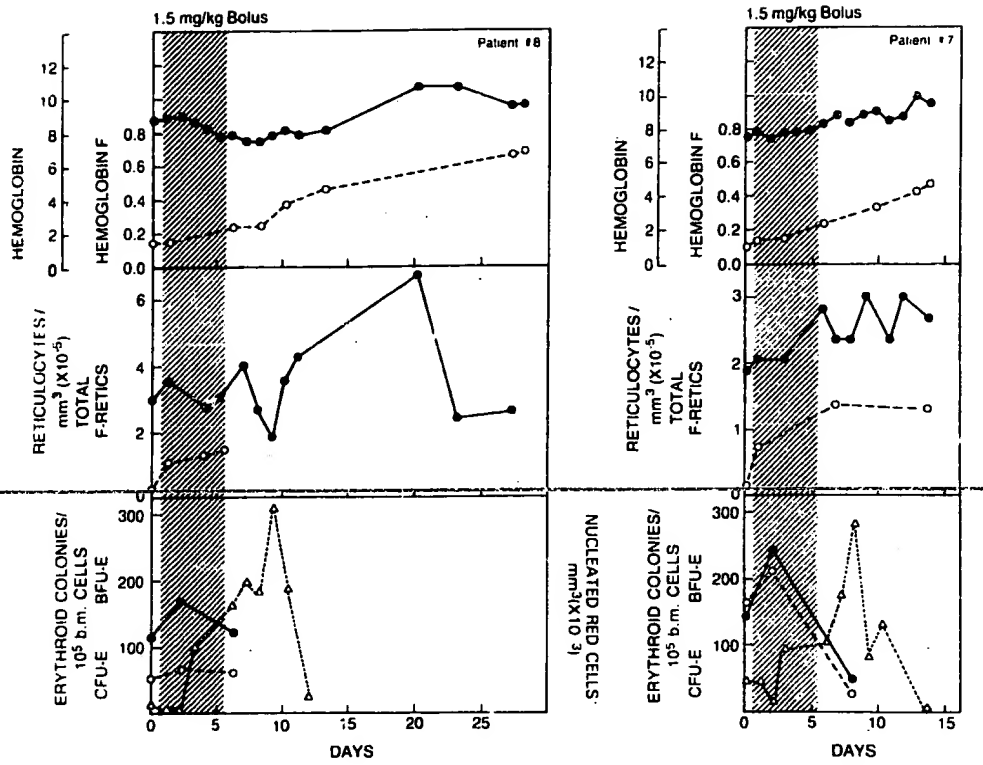

Figure 7. Clinical courses of patients 8 (*left*) and 7 (*right*) on treatment with 5-azacytidine. The periods of 5-azacytidine administration are shaded. The hemoglobin (*g/dl*, — ● —) and hemoglobin F level (*g/dl*, - - - o - - -), total (— ● —), and F-reticulocyte (- - - o - - -) counts, nucleated erythrocytes (- - - Δ - - -), and erythroid colonies per 10^5 bone marrow cells (CFU-E, — ● —; and BFU-E, - - - o - - -) are plotted for the patient indicated.

base-line studies. No cultures corresponding to our day 2 studies were obtained during 5-azacytidine administration. Torrealba-de Ron et al. (19) suggested that these toxic effects on late erythroid progenitors and the subsequent regeneration of these late progenitors from earlier progenitors were responsible, at least in part, for the augmentation in synthesis of HbF. A shift to a less mature progenitor as a source of erythroblasts and ultimately reticulocytes has also been proposed as the basis for the ability of hydroxyurea to augment HbF synthesis in anemic monkeys (*Macaca fascicularis*) (20).

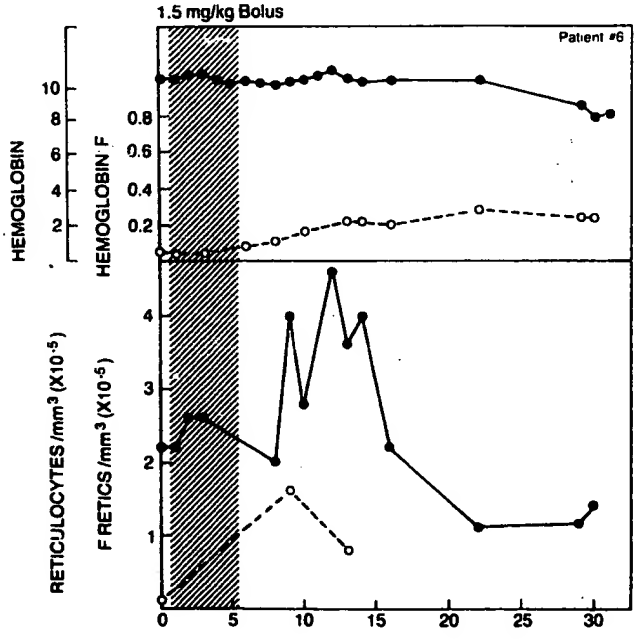

Figure 8. Clinical course of a moderate responder (patient 6) to treatment with 5-azacytidine. Shown are the total (*g/dl*, — ● —) and F hemoglobin (*g/dl*, - - - o - - -) levels, and total (— ● —) and F-reticulocyte (- - - o - - -) counts during (shaded area) and after administration of 5-azacytidine.

We interpret our analysis of the kinetics of the effect of 5-azacytidine on human erythroid cells as being most consistent with a direct action of 5-azacytidine on erythroblasts; moreover, a toxic or cytoreductive action on late progenitors is not necessary to achieve an increase in HbF synthesis in the erythroblasts derived from these cells. Strongly supporting a direct action are the early rise in F-reticulocytes and γ-mRNA levels reported herein and in previous studies (16–18). For example, patients 7 and 8 exhibited a two to threefold increase in the percentage of F-reticulocytes within 24–48 h of starting 5-azacytidine treatment (Fig. 4) without a significant change in total reticulocyte concentration. Ferrokinetic studies (33) and ^{59}Fe-labeling of bone marrow cells (34) suggest a transit time from bone marrow proerythroblasts to peripheral blood reticulocytes of from 72 to 95 h. Although this interval might be shortened in the presence of hemolysis, accelerated erythropoiesis, and drug administration, it seems unlikely that reticulocyte counts could be sustained at relatively constant levels during 5-azacytidine administration by recruitment and accelerated maturation of erythroid progenitors if there were substantial killing of cells already in the erythroblast compartment. The mechanism of this early effect on the erythroblast population remains conjectural but presumably must depend on some distortion of the normal erythroid maturational kinetics.

48 h after treatment is started, hypomethylation of a CpG residue immediately upstream from the γ-gene coding se-

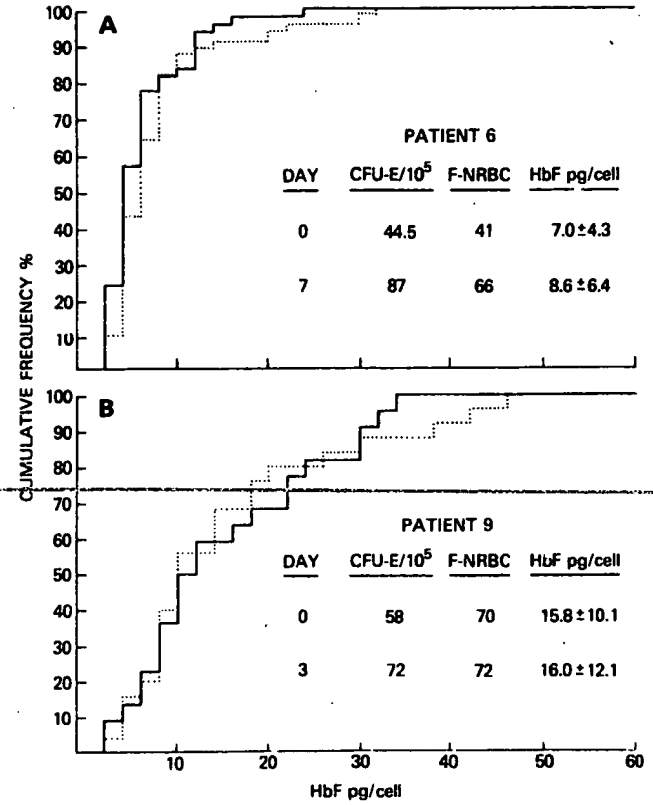

Figure 9. HbF content of individual nucleated erythrocytes derived in vitro from CFU-E obtained from two patients who received 5-azacytidine. Well-hemoglobinized colonies derived from CFU-E were plucked on the eighth day of in vitro culture and HbF content of individual erythroblasts was determined by radial immunodiffusion. Note that colony erythroblasts contain 40–60 pg of total hemoglobin. *A* depicts the results from patient 6 (see Table I) and *B* depicts the results from patient 9 during his second treatment course (see Table I). Data are presented as cumulative frequency plots of erythroblasts containing up to a certain amount of HbF. Determinations were made on bone marrow samples obtained at the start of 5-azacytidine treatment (day 0, ——) and 3 or 7 d later (· · · · ·).

quences is demonstrable in the bone marrow cells of most patients (35). However, the causal role of demethylation in inducing gene expression has been questioned (36, 37). Indeed, the ability of 5-azacytidine to specifically turn on the γ-gene but not other genes is not readily explained, although it may be argued that hypomethylation is necessary but insufficient for gene activation. Indeed, failure of patient 9 to show an increase in HbF synthesis despite demonstrated hypomethylation of γ-gene sequences supports this interpretation. Recently, activation of the γ-globin gene but not the genes for ε-globin or insulin, has been shown to occur after treatment of somatic hybrid cells with 5-azacytidine in vitro (38). These hybrid cells, derived by fusion of mouse erythroleukemia cells and human fibroblasts containing a chromosome 9-11 translocation, usually express only the human β-globin gene after induction to erythroid maturation. The selective activation of the γ-gene in these cells by 5-azacytidine, despite generalized DNA hypomethylation, indicates that this restricted effect on the γ-gene can occur outside of the normal pathway of erythroid progenitor and precursor maturation.

An alternative mechanism by which 5-azacytidine could enhance globin production in erythroblasts relates to the known asynchronous synthesis of HbF and HbA during erythroid maturation (2–4). HbF synthesis apparently occurs mainly in pro- and basophilic erythroblasts; HbA synthesis begins slightly later in the maturation pathway but greatly exceeds HbF synthesis during the later stages of erythroblast maturation. Cell-cycle specific agents such as 5-azacytidine might alter the kinetics of cell division and delay the normal switch from HbF to HbA during cellular maturation in the erythroblast compartment. To account for the increased percentage of F-reticulocytes by this mechanism would require that drug administration also increase the proportion of erythroblasts that make HbF.

Erythroid progenitors exposed to 5-azacytidine either in vivo or in vitro yielded colonies that made more HbF than did control progenitors, even though colony formation occurred in the absence of the drug. Moreover, the number of progenitors detected was stable or even increased after short exposure to

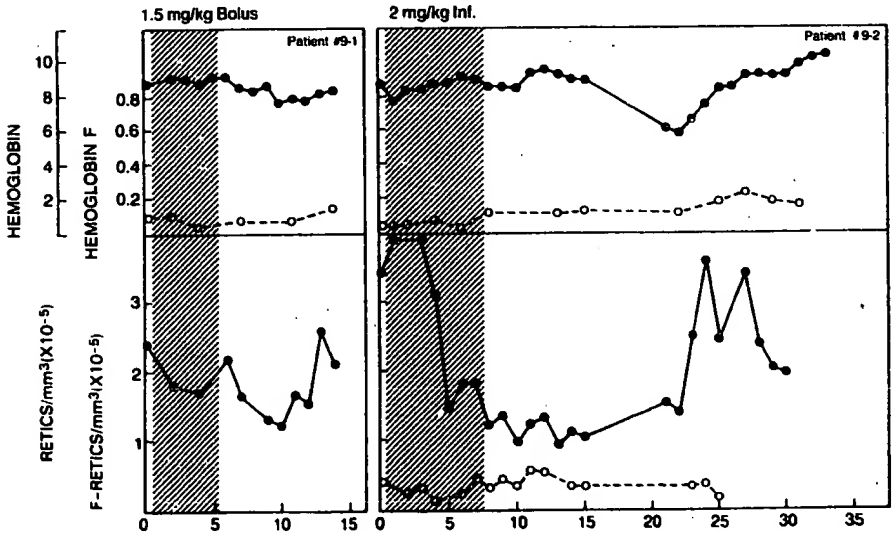

Figure 10. Clinical course of a nonresponder (patient 9) during two courses of 5-azacytidine treatment. Shown are total hemoglobin (*g/dl*, — ● —) and HgF (- - - ○ - - -) levels, and total (— ● —) and F-reticulocytes (- - - ○ - - -) over the course of two 5-azacytidine treatment regimens (see Table I). The period of administration of 5-azacytidine is indicated by the shaded area.

5-azacytidine. Taking these results together we argue that the initial effect of 5-azacytidine on the erythroid progenitor compartment observed after 2 d of drug administration and the subsequent increase in HbF synthesis do not occur by significant cytoreduction and then regeneration of erythroid progenitors. Rather, 5-azacytidine leads to progenitors committed to increased HbF production.

The possible mechanisms for this early effect on erythroid colony-forming cells are (*a*) reprogramming of individual erythroid progenitors, perhaps by a demethylating effect, or (*b*) recruitment of erythroid progenitors that normally would not contribute to the pool of maturing erythroid precursors. We cannot exclude the possibility of a rapid cytotoxic action with a rapid recovery in vivo since time points for bone marrow sampling were necessarily limited. Definitive tests of these possibilities await the availability of purified populations of erythroid progenitors where reprogramming, recruitment, and cytotoxicity can be rigorously assessed. We observed variable toxicity on the late erythroid progenitor cells in cultures of bone marrow obtained on day 7 of the treatment schedule analogous to the decrease in colony forming progenitors in bone marrows from baboons on day 6 of their treatment schedule with 5-azacytidine (19). Evidence of cytotoxicity should not be used to infer that this is necessarily the mechanism by which the drug works. Our data seem most consistent with the interpretation that this late toxic effect is neither crucial for, nor consistent with, the kinetics of the observed increase in HbF synthesis. The direct demethylating effect of 5-azacytidine is not required since hydroxyurea (20) and arabinosylcytosine (39) also lead to increased HbF synthesis. The data seem most consistent with a recruitment model analogous to that proposed by Papayannopoulou et al. (39) although we disagree with their interpretation that significant cytoreduction is required.

The previously reported (18) increase in nucleated erythrocytes in the peripheral blood of sickle cell anemia patients between 6 and 10 d after treatment with 5-azacytidine is initiated has proven to be a consistent effect, although it does not occur in patients with thalassemia. We understand neither the mechanism of this effect nor the reason why it is restricted to those patients with sickle cell anemia. The increase in nucleated erythrocyte count follows the increase in assayable erythroid progenitors by 4–8 d. Perhaps the properties of erythroid precursors derived from the early recruited progenitors are altered in a way that allows their accelerated release from the bone marrow.

Our experience has revealed considerable heterogeneity in the clinical response to 5-azacytidine. Many patients show an early rise in F-reticulocytes and a sustained increased in γ-globin gene expression, leading to a progressive five to sixfold increase in HbF and a steady rise in hemoglobin concentration (Fig. 7). Other patients show only a moderate increase in gene expression and HbF concentration but little or no increase in hemoglobin concentration (Fig. 8). In a minority of patients with sickle cell anemia, HbF containing erythrocytes do not appear to have prolonged survival as compared with non-HbF containing cells (40), which perhaps accounts for the failure of the hemoglobin concentration to rise in some patients treated with 5-azacytidine.

One of the nine patients showed no significant increase in γ-gene expression, as assessed by enumeration of F-reticulocytes or measurement of γ-mRNA concentration in bone marrow. Selective toxicity to these cells producing only HbA resulted in a substantial fall in total reticulocytes but a constant F-reticulocyte concentration led to a modest increase in HbF concentration in blood. The kinetics of this response were quite different from those of the response exhibited by patients who have a substantial increase in γ-gene expression as judged by the F-reticulocyte increment and an increase in γ-mRNA concentration in bone marrow (compare Fig. 7 with Fig. 10).

5-Azacytidine appeared to hold some promise for the treatment of patients with sickle cell disease. Charache and Dover have treated three patients for extended periods and observed a persistent increase in HbF concentration and some clinical improvement (21). A controlled trial could now be justified and would be required to determine whether significant clinical benefit could be achieved with the drug. For the present, however, use of 5-azacytidine in humans has been discontinued. This decision is based on concern about its potential carcinogenic effect and because cytosine arabinoside (39) and hydroxyurea (20) have also been shown to increase HbF synthesis. These drugs, particularly hydroxyurea, are thought to be less carcinogenic. That low doses of 5-azacytidine increase HbF synthesis without depressing the bone marrow may have important implications for the choice of dose and schedule of administration of other drugs, given in an effort to achieve a clinically useful increase in production of HbF.

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TI 5-Azacytidine acts directly on both erythroid precursors and progenitors to increase production of fetal hemoglobin
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Pharmacological Induction of Fetal Hemoglobin in Sickle Cell Disease and β-Thalassemia

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A number of pharmacological agents are currently available for the induction of fetal hemoglobin (HbF) in patients with sickle cell disease and β-thalassemia. Here we review the development of this new class of therapeutics and summarize the clinical trials that investigate their efficacy in patients with hemoglobin disorders. Hydroxyurea is the first of these drugs to be approved by the Food and Drug Administration for the treatment of sickle cell disease. Currently, the major focus is the development of safer agents and combinations of drugs that can increase HbF to levels high enough to prevent all complications of the disease. Progress in adapting the same strategy to the treatment of thalassemic disorders has been much slower. Although all the agents that are effective in sickle cell disease have similar HbF-inducing activity in β-thalassemia, their use has rarely resulted in significant amelioration of the anemia. More research and more effective agents will be needed to make a significant impact on thalassemia. Nonetheless, success in this relatively young field has been very gratifying; before the end of this decade, clinically meaningful induction of HbF may become an achievable goal in most patients with hemoglobin disorders.

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THE BENEFICIAL EFFECTS of high levels of fetal hemoglobin (HbF) in sickle cell disease and β-thalassemia have been recognized for many years. In 1948, Watson et al noted that newborns with sickle cell disease do not manifest significant clinical problems related to their disease in the first 6 months of life, before the HbF declines to adult levels.[58] It was later shown that most patients with sickle cell disease from certain regions of Saudi Arabia[37] and India[5] who inherit a genetic determinant for high HbF have a very mild sickling disorder. More recently, the Cooperative Study of Sickle Cell Disease, a large multicenter examination of the natural history of sickle cell disease, demonstrated an inverse correlation between HbF levels and the frequency of painful crises and early death.[41,43] These clinical and epidemiological observations were also supported by laboratory findings of a sparing effect of HbF on polymerization of deoxyhemoglobin S.[44] Similarly, an increase in the synthesis of fetal γ-globin chains can decrease the imbalance between α- and non–α-globin chains in β-thalassemia and ameliorate the severity of the anemia. This appreciation of the beneficial effects of HbF in sickle cell disease and β-thalassemia has stimulated interest in the development of therapeutic agents that can increase HbF production in patients with these disorders.

Induction of HbF in Sickle Cell Disease

5-Azacytidine

5-Azacytidine was the first HbF-inducing agent to be used therapeutically in patients with sickle cell disease. Its DNA-demethylating activity was hypothesized to result in hypomethylation of the promoters of the fetal γ-globin genes and their transcriptional activation. Earlier molecular studies had demonstrated that the promoters of the γ-globin genes were hypomethylated when the genes are expressed in fetal life and hypermethylated when they are silenced in adult life.[50] The induction of HbF by 5-azacytidine was first seen in phlebotomized baboons[13] and later in patients with sickle cell disease and β-thalassemia.[28,33] Although these small clinical trials generated considerable enthusiasm, concern about potential short- and long-term toxicity of a DNA-damaging chemotherapeutic agent like 5-azacytidine prevented its widespread use in patients with hemoglobin disorders.

Hydroxyurea

Interestingly, controversy about the mechanism of activation of fetal γ-globin gene expression by 5-azacytidine led to the identification of hydroxyurea as a second therapeutic agent that can stimulate HbF production. Some investigators postulated that 5-azacytidine increased HbF production by accelerating erythroid cell differentiation rather than by hypomethylation of DNA.[27] To support this view, experi-

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ments were performed to show that hydroxyurea, an S-phase–specific chemotherapeutic agent with no DNA demethylating activity, can also increase HbF production in phlebotomized baboons[27] and in patients with sickle cell disease.[42] These observations, however, did not completely resolve the mechanism of action, since 5-azacytidine appears to be a more potent inducer of HbF production than is hydroxyurea. Moreover, a recent study demonstrated that a new 5-azacytidine analog, 2-deoxy-5-azacytidine, can induce HbF in sickle cell patients who are resistant to hydroxyurea, suggesting that the two drugs have different activities.[25] In contrast to 5-azacytidine, hydroxyurea is an oral agent with a relatively good safety profile, and it is already widely used in the treatment of myeloproliferative disorders. Thus, hydroxyurea quickly replaced 5-azacytidine as the drug of choice for stimulating HbF production.

Hydroxyurea was first used in a number of small nonrandomized clinical trials that confirmed its HbF-inducing activity in sickle cell disease[8,42,48] and led to an effective dose schedule for treatment of patients. The phase I/II studies were followed by a large randomized, placebo-controlled Multicenter Study of Hydroxyurea (MSH), which had well-defined clinical efficacy endpoints.[9] This trial was terminated prematurely when interim analysis showed a significant reduction in the frequency of crises and acute chest syndrome in the hydroxyurea-treated group compared to controls. There was also a reduction in the frequency of blood transfusions in the hydroxyurea-treated patients but no changes in the rate of stroke or death during the relatively short duration of the study.[9] On the basis of these findings, hydroxyurea became the first HbF-inducing drug to be approved by the Food and Drug Administration for the treatment of sickle cell disease. As a result, hydroxyurea is now widely used in the United States and Europe for this indication.

Since the first MSH report of clinical efficacy of hydroxyurea in sickle cell disease, detailed analyses of other clinical and laboratory data that were collected as part of the MSH have been published.[7,53] Curiously, one of these studies failed to show a correlation between the increase in HbF production and clinical benefit in patients who received hydroxyurea[7]; a correlation was identified between the decrease in white blood cell (WBC) count and efficacy. This surprising finding led to widespread speculation that the therapeutic effects of hydroxyurea were related to its effect on WBCs rather than to increased HbF production. We caution against such a premature conclusion for three reasons. First, MSH was not designed to investigate the mechanism of action of hydroxyurea. Second, HbF levels were measured only at the beginning and end of the study. Since not all HbF levels were available for analysis, the F-cell number was used as a surrogate. However, this number may not be a good replacement for HbF levels, since the fold increase in HbF levels is greater than the fold increase in F cells in patients treated with hydroxyurea or other HbF-inducing agents.[6] Third, a recent follow-up of the same population of patients who were originally enrolled in MSH did show a positive correlation between survival and HbF levels in those who were treated with hydroxyurea.[52]

Butyrates

In 1985, Ginder et al reported that the administration of butyrate to chickens pretreated with 5-azacytidine resulted in the induction of embryonic globin gene expression.[19] Others then showed that infants born to diabetic mothers had higher HbF levels at birth than age-matched controls; the diabetic pregnant mothers had high levels of butyric acid in their plasma. Butyrate infusions in utero in sheep fetuses prevented the switch from fetal to adult hemoglobin normally seen around the time of birth,[40] and the administration of butyric acid to adult baboons could partially reverse the switch from fetal to adult globin expression.[12] These preclinical observations formed the basis for clinical trials that investigated the therapeutic potential of butyrate as a HbF-inducing agent in patients with sickle cell disease and β-thalassemia.

Perrine et al first evaluated the effects of arginine butyrate on HbF production in six patients with β-globin disorders (three with sickle cell disease and three with thalassemia). Butyrate resulted in an increase in γ-globin chain synthesis in all six cases following a 2-week infusion of the drug.[38] When arginine butyrate infusions were given to five patients with sickle cell disease and five with β-thalassemia, a 10-week dose escalation course resulted in a significant increase in HbF levels in two of the five sickle cell patients.[51] This improvement in HbF levels, however, was not sustained with continuous high-dose therapy, and there was no increase in the total hemoglobin levels in any of the five β-thalassemia patients.

Sodium phenylbutyrate was also used to induce HbF production in sickle cell disease. All six patients who received the drug orally showed a rapid increase in the number of circulating F reticulocytes.[15] Two of the six patients who received the drug for 5 to 6 months increased their HbF levels from 11% to 18% and from 10% to 16%, respectively. However, since this oral regimen required the intake of 30 to 40 tablets per day, poor compliance limited its effectiveness in the outpatient setting.

The potential role of intravenous arginine butyrate in sickle cell disease has recently been revisited.[3] The first six patients who were enrolled in this new study received arginine butyrate infusions for 8 hours per day, 5 days per week. HbF levels increased in half of

these patients but were not sustained with continuous long-term therapy. These observations and those summarized above[51] suggested that toxicity resulting from long-term exposure to butyrate might be responsible for the loss of the HbF response. A regimen in which butyrate is given intermittently to allow recovery of the bone marrow from butyrate's antiproliferative effects was then investigated. The HbF levels of 11 patients enrolled on this regimen increased from a mean of 7% at baseline to 21% on intermittent butyrate therapy; this response persisted in all patients, including one who has received arginine butyrate for more than 6 years.[3]

All five patients who did not respond to both continuous and intermittent butyrate had baseline HbF levels below 2%, while all 10 responders had baseline HbF levels of 2% or above.[3] Although the exact mechanism of stimulation of HbF production has yet to be determined, it is believed that butyrate's effect is at least in part mediated by inhibition of histone deacetylases. Inhibition of histone deacetylases results in increased acetylation of chromatin, which makes DNA more accessible to transcription factors. This mechanism, however, does not provide any insight into the molecular basis for the specificity of the γ-globin gene activation effect of butyrate. More recently, it has been recognized that histone deacetylases can act regionally through their recruitment to DNA in a sequence-specific manner by binding to transcription factor complexes,[21,26] explaining the specificity of butyrate and the clinical observation that its HbF-inducing activity requires partially active human γ-globin genes, whose regulatory elements may already be occupied by transcription factors.

Combination Therapy

Of the five patients in the study summarized above who did not respond to butyrate, three later were treated with hydroxyurea: all increased their HbF levels above 20%. Conversely, a majority of patients who failed to respond to hydroxyurea increased their HbF levels in response to 2-deoxy-5-azacytidine.[25] This absence of cross-resistance to the HbF-inducing activities of 5-azacytidine, hydroxyurea, and butyrate confirms that these drugs activate γ-globin gene expression by different mechanisms. We proposed the hypothesis that the use of these agents in combination regimens could induce HbF production in an additive or synergistic manner. This hypothesis has recently been tested in three patients enrolled on a protocol consisting of hydroxyurea for several months followed by hydroxyurea and butyrate. All showed marked increases in their HbF levels after butyrate was added to hydroxyurea. Thus, the combination of butyrate and hydroxyurea was more effective than hydroxyurea alone. In one patient who was totally resistant to the effect of butyrate following both weekly and intermittent therapy, addition of butyrate after hydroxyurea therapy resulted in a large increment in the HbF level. Thus resistance to butyrate was not absolute and could be reversed following pretreatment with hydroxyurea.[54] Similar advantages of combinations of other HbF-inducing agents were previously described in studies of hydroxyurea with erythropoietin[33] and hydroxyurea with sodium phenylbutyrate.[35]

Before embarking on large studies of combinations of HbF-inducing agents, it is important to ask if there is a need for the use of two or more agents in sickle cell disease. Natural history study of sickle cell disease had clearly shown that higher HbF levels are associated with a less severe clinical outcome,[41,43] but the clinical benefits of increased HbF levels may plateau when values exceed 20%. Even though patients from Saudi Arabia and India, whose HbF levels are generally above 20%, have a very mild clinical course, they are not always free of sickling complications.[3] One sickle cell disease patient developed progressive pulmonary hypertension while her HbF level was greater than 20% on hydroxyurea; the addition of butyrate in this case resulted in a peak HbF level of 45% and marked amelioration of the pulmonary hypertension.[55] Although there is no definitive evidence yet that HbF levels of 30% to 45% are significantly better than 20% to 30%, laboratory data support the hypothesis that such high levels may be necessary to completely inhibit intracellular polymerization of deoxyhemoglobin S in red blood cells.[34] Thus, with the current availability of multiple drugs that can activate HbF additively or synergistically, we suggest that the aim of pharmacologic therapy should be to achieve the highest possible HbF level, rather than to be satisfied with the traditionally accepted level of 20%.

In addition to drugs that stimulate HbF production, a number of other agents that target different aspects of the pathophysiology of sickle cell disease are under investigation. These include antiadhesive agents, antidehydration drugs, and possibly anti-WBC agents. If they prove to be effective in clinical trials, it would be of considerable interest to investigate combinations of HbF-inducing agents with pharmaceuticals for different aspects of the pathophysiology of sickle cell disease. After more than 50 years of intense research, we might soon be in a position to select, based on the genotype and/or phenotype of a patient, one or more therapeutic agents for different aspects of the pathophysiology of sickling. It is gratifying that the intense study of the molecular and cellular basis of this disease is finally being translated into novel therapies to impact on the lives of patients.

Induction of HbF in the β-Thalassemia Syndromes

All of the pharmacological agents that have been investigated for their HbF-inducing activity in sickle cell disease have also been tested in β-thalassemia. Although HbF-inducing activity also has been seen in thalassemia, meaningful hematological and/or clinical responses have been much more difficult to achieve. HbF induction in the β-thalassemia syndromes has been a therapeutic challenge and it remains uncertain whether this therapeutic approach will eventually have an impact on the treatment of this disorder.

Hydroxyurea

Hydroxyurea in thalassemia major. The first study of hydroxyurea in thalassemia major was published in 1985; administration of the drug to six transfusion-dependent patients did not lead to a reduction in the frequency of transfusion.[33] A better response was observed in a clinical trial in India, where hydroxyurea in 15 multitransfused thalassemic children resulted in a (statistically insignificant) increase in the levels of total hemoglobin and HbF in the majority of cases.[10] The discrepancy between these results could be a reflection of real differences in the effectiveness of hydroxyurea in thalassemia deriving from distinct molecular defects. When hydroxyurea was used to relieve neurologic abnormalities produced by extramedullary erythropoietic masses in a patient with Hb Lepore/β-thalassemia, there was a dramatic increase in both total hemoglobin (from 5.8 to 9.7 g/dL) and HbF (from 4.9 to 9.1 g/dL).[47] (Other studies of hydroxyurea have shown efficacy in the treatment of nervous system complications in this setting.[18,23]) Alternatively, chronic transfusions in patients with β-thalassemia major may prevent HbF induction by hydroxyurea: in a 20-year old alloimmunized patient who could not be transfused, hydroxyurea produced a dramatic increase in the total hemoglobin level to about 10 g/dL.[2] Similar observations in sickle cell disease suggest that blood transfusions can result in a loss of the HbF response to hydroxyurea, butyrate, and/or combinations of hydroxyurea and butyrate (unpublished observations, G.F.A.).

Hydroxyurea in thalassemia intermedia. The effects of hydroxyurea in thalassemia intermedia are not obscured by blood transfusions. Moreover, most patients with thalassemia intermedia have low levels of HbF in their red blood cells, so it is easier to detect the effect of hydroxyurea. The administration of hydroxyurea to a nontransfused patient with thalassemia resulted in a significant increase of total hemoglobin from 4.6 to 6.6 g/dL.[33] Three other patients with thalassemia intermedia responded to hydroxyurea with a transient increment of total hemoglobin and a significant increase in HbF levels[20]; a larger number of similar patients treated with hydroxyurea for 16 to 24 months showed the same results.[30,56] In a prospective controlled study, patients were divided into two groups: group A consisted of five patients with thalassemia intermedia who received hydroxyurea at 1.0 to 1.5 g/d and group B of five patients treated at 0.3 to 0.5 g/d.[56] A few weeks after initiation of therapy, patients in group A showed a significant increase in HbF along with a lower number of normoblasts and reticulocytes in their peripheral blood. However, total hemoglobin levels were virtually unchanged. Mean corpuscular volume and mean corpuscular hemoglobin were significantly increased, while mean corpuscular hemoglobin concentration remained the same. The increase in HbF production was associated with an increase in the ratio of $\gamma/\beta-+\gamma$ chain biosynthesis. Serum erythropoietin levels did not significantly change, while soluble serum transferrin receptor (sTfR) levels decreased considerably. In contrast, patients in group B did not show any alterations in their total hemoglobin or HbF levels and their sTfR levels remained stable. Nevertheless, a significant increase of the β-/α-globin chain biosynthetic ratios along with a rise in total hemoglobin levels was seen in two Chinese patients with β-thalassemia treated with low-dose hydroxyurea (0.2 to 0.3 g/d).[59] It is not clear why the Greek and Chinese patients responded differently at these low doses. Erythroid cells in culture showed increased expression of γ-mRNA in Greek patients with thalassemia,[22] while for the Chinese patients with β-thalassemia β-globin mRNA expression in their erythroid cells in culture increased following exposure to hydroxyurea.[59]

Hydroxyurea in β-thalassemia/hemo-globin E disease. When hydroxyurea was administered to 13 patients with hemoglobin E (HbE)/β-thalassemia, multiple effects were observed: (1) a significant increase in HbF levels in all cases (from a mean of 42% ± 11% to 56% ± 8%); (2) a significant improvement in the ration of α-/non–α-globin chain synthesis; (3) a 10% increase in hemoglobin levels; and (4) a decrease in circulating reticulocytes along with almost complete disappearance of normoblasts from the peripheral blood. Taken together, these results suggest that more effective erythropoiesis may have resulted from the selective proliferation and survival of HbF-containing erythroid cells.[17]

Hydroxyurea in sickle cell/β-thalassemia. The study of hydroxyurea in compound heterozygotes for hemoglobin S (HbS)/β-thalassemia in Mediterranean countries started soon after the initial reports of its benefits in patients homozygous for the β^S gene. The severity of the HbS/β-thalassemia syndrome can vary enormously in different populations.[29] In the Medi-

terranean, the most common β-thalassemia genes (β^0-thalassemia [CD39 C to T or IVS1-nt1 G to A] or β^+-thalassemia [IVS1-n110 G to A or IVS2-nt 745 C to G]) do not result in the production of the high levels of hemoglobin A that can prevent sickling, as in African American patients who commonly have thalassemic genes with milder defects.[29] The benefit of hydroxyurea in HbS/β-thalassemia is not limited to the prevention of vaso-occlusive crises through prevention of the polymerization of HbS, and possibly also the inhibition of cell adhesion. Hydroxyurea neutralizes the catastrophic effects of excess α-globin chains. Loukopoulos et al have administered hydroxyurea over the past 10 years to a relatively large cohort under close observation (79 patients to date).[31,36] Most received hydroxyurea at 1.5 g/d, with relatively few side effects and a good outcome. Prior to starting therapy, the treating physicians assessed quantitatively, on an arbitrary numeric scale, the impact of disease in terms of crises leading to hospitalization, pain requiring analgesics at home, and discomfort relieved with simple analgesics but still interfering seriously with the patients' quality of life. The same scale was used during their enrollment in the clinical trial. The total score of the 59 evaluable patients for the 52 weeks preceding treatment (3,068 patient-weeks) was 1,182 points; the total score for the same patients during enrollment into the study (12,018 patient-weeks) was only 82 points. There was significant improvement of the severe hepatic cholestasis occurring in some sickle cell disease patients. The development of leg ulcers was possibly related to treatment with hydroxyurea. Additional findings included (1) a dramatic increase in HbF levels (up to 40% in some cases); (2) a slight increase in total hemoglobin in male patients with HbS/β^0-thalassemia (from 8.7 ± 1.4 to 10.1 ± 2.8 g/dL); (3) an increase in the red blood cell indices; (4) a significant decrease of the sTfR (six of seven patients); and (5) a surprisingly large increase in serum erythropoietin levels a few weeks after initiation of treatment.[36] Results from other studies are similar, but because of the small numbers of compound heterozygotes treated with hydroxyurea, the data are usually included with those of patients homozygous for the sickle gene.[16,24]

Erythropoietin With or Without Hydroxyurea in β-Thalassemia Intermedia

The effect of erythropoietin as a single agent has been investigated in patients with β-thalassemia intermedia. The hormone produces an increase in "thalassemic erythropoiesis" without corresponding improvement in levels of HbF. A significant increase in total hemoglobin levels occurred only in splenectomized patients.[46] The combination of hydroxyurea and recombinant erythropoietin can additively increase F-cell numbers in baboons.[1] A small study in patients with β-thalassemia using this combination also showed that the two agents can have an additive effect, resulting in increased HbF and total hemoglobin levels.[45] In another short clinical trial, in which 10 patients with thalassemia intermedia were given hydroxyurea in combination with recombinant human erythropoietin subcutaneously for 12 weeks, eight responded with a significant increase of their hemoglobin levels, and half of the responders also had a parallel increase in the level of HbF; the effects persisted for the duration of therapy.[30,32]

Butyrates

As mentioned above, the majority of studies of HbF induction by butyrates included patients with β-thalassemia as well as sickle cell disease,[38,51] and evidence for HbF-inducing activity was seen in both syndromes.[38] The major endpoint of induction of HbF in sickle cell disease is the percentage HbF in the red cells of the peripheral blood. In contrast, the major endpoint of induction of HbF in β-thalassemia is the total hemoglobin level. As for hydroxyurea, although an increase in HbF levels is frequently seen in patients with β-thalassemia following butyrate therapy, an increase in total hemoglobin levels has been much more difficult to achieve.[51] One notable exception is a patient homozygous for Hb Lepore who had a very significant rise in total hemoglobin levels in response to arginine butyrate[38] and to a combination of hydroxyurea and oral sodium phenylbutyrate.[35] Four of eight nontransfused patients with β-thalassemia intermedia treated with oral phenylbutyrate increased their total hemoglobin by a mean of 2.1 g/dL.[11] In Italian patients with β-thalassemia intermedia treated with isobutyramide, another orally bioavailable short-chain fatty acid, the drug clearly had some activity, but the dose regimen did not result in significant and sustained correction of the anemia.[14] Intermittent arginine butyrate (with or without erythropoietin) is currently under investigation in patients with β-thalassemia intermedia (S. Perrine, personal communication), but it is too early to determine whether this schedule will be as effective in thalassemia as in sickle cell disease.

Conclusions

The idea of selective induction of HbF production in patients with sickle cell disease and thalassemia was only a dream as recently as two decades ago. Such treatment has already become feasible and has made a major impact on the clinical course of sickle cell disease. Treatment with hydroxyurea has had a significant effect on the quality of life and possibly also

on long-term survival in sickle cell patients. The future holds considerable promise for developing safer and even more effective agents for the induction of HbF in sickle cell patients. Using combinations of drugs, it may be possible to increase HbF levels sufficiently to prevent all short- and long-term complications of the disease. So far, the outlook for success of HbF induction therapy in the thalassemia syndromes has not been as bright. That a side effect of most of the currently available pharmacologic agents is suppression of erythropoiesis may make it difficult to correct the anemia in spite of correction of the ineffective erythropoiesis. The inhibitory effect of transfusions on responsiveness to these pharmaceuticals has been a challenge in thalassemia major. The success of this therapeutic approach in β-thalassemia may be possible with refinements in the schedule of administration of these agents to allow sufficient induction of HbF production while minimizing bone marrow suppressive effects. Alternatively, the development of a new class of agents that can induce HbF production and at the same time stimulate erythropoiesis may be necessary. Novel molecules currently are being investigated in the laboratory[57] and their transition to the clinic may make it possible to develop effective HbF induction therapy for all the thalassemia syndromes.

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AN 136:128460 CA
TI Pharmacological induction of fetal hemoglobin in sickle cell disease and .beta.-thalassemia
AU Atweh, George F.; Loukopoulos, Dimitris
SO Seminars in Hematology (2001), 38(4), 367-373
CODEN: SEHEA3; ISSN: 0037-1963

L10 ANSWER 7 OF 14 CA COPYRIGHT 2003 ACS
AN 103:20543 CA
TI Induction of hemoglobin F synthesis in patients with .beta. thalassemia
AU Ley, Timothy J.; Nienhuis, Arthur W.
SO Annual Review of Medicine (1985), 36, 485-98

L10 ANSWER 8 OF 14 CA COPYRIGHT 2003 ACS
AN 102:125352 CA
TI 5-Azacytidine acts directly on both erythroid precursors and progenitors to increase production of fetal hemoglobin
AU Humphries, R. Keith; Dover, George; Young, Neal S.; Moore, Jeffrey G.; Charache, Samuel; Ley, Timothy; Nienhuis, Arthur W.
SO Journal of Clinical Investigation (1985), 75(2), 547-57

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Ann. Rev. Med. 1985. 36 : 485-98

INDUCTION OF HEMOGLOBIN F SYNTHESIS IN PATIENTS WITH β THALASSEMIA[1]

Timothy J. Ley, M.D. and Arthur W. Nienhuis, M.D.

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ABSTRACT

5-Azacytidine transiently augments fetal hemoglobin production in patients with β-thalassemia or sickle cell anemia. This change would probably be beneficial to such patients (e.g. a normal fetal gene product is substituted for a deficient or defective adult gene product) if HbF production could be sustained at high levels for prolonged periods. Even though the clinical use of 5-azacytidine is limited because of its presumed potential to cause cancer, studies with this drug have provided new insights into globin gene regulation and have stimulated the development of other strategies to increase HbF synthesis.

INTRODUCTION

Severe β thalassemia is a disease of world-wide importance, affecting tens of thousands of people of Mediterranean, Middle Eastern, Indian, and Southeast Asian descent (1). This disease is caused by mutations that affect the ability of a β globin gene to produce a normal quantity of its messenger RNA (mRNA) and protein. People with a defect in only one β globin gene may have a mild anemia with hypochromic microcytic red blood cells, or may have no hematologic abnormalities at all. Patients with defects in both β globin genes have homozygous thalassemia, and may either be moderately anemic (thalassemia intermedia) or severely anemic (thalassemia major).

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Patients homozygous for β thalassemia mutations generally require transfusions beginning in early infancy. Transfusions are required in some to sustain life, and in all to prevent the skeletal abnormalities that accompany extreme bone marrow expansion. However, the consequence of sustained transfusional support is the inevitable deposition of iron in the heart and other organs, a complication that generally leads to the patient's death during the second or third decade (2). Early initiation of chelation therapy with deferoxamine may well forestall the consequences of iron overload (3), but more definitive forms of treatment are needed.

During the past few years, the human α-like and β-like globin genes have been cloned and their sequences determined. Most of the genetic lesions that cause β thalassemia have subsequently been characterized (1); in addition, the function of normal and thalassemic genes has been carefully examined in vitro. Study of these genes revealed a large variety of mutations, and added greatly to our understanding of eukaryotic gene function. Furthermore, these studies provided new strategies for the therapy of genetic diseases, including reactivation of fetal hemoglobin (HbF) synthesis in β thalassemia. This review describes normal and abnormal globin gene physiology, and outlines the rationale and results of attempts to augment HbF synthesis in patients with β thalassemia and sickle cell anemia.

NORMAL AND THALASSEMIC GLOBIN GENE FUNCTION

In normal adults, two α globin genes lie on each chromosome 16, and one β globin gene is found on each chromosome 11 (see Figure 1, top panel). The four α genes produce about 40% more mRNA than the two β genes; however, since β mRNA is translated more efficiently than α, and since some α globin is degraded, a balanced number of α and β globin protein molecules normally exists in each adult red cell (4). The α and β chains combine stoichiometrically with heme to form the tetrameric compound HbA ($\alpha_2\beta_2$).

In patients with homozygous β thalassemia, the output of the two β globin genes is markedly reduced (β^+) or absent (β^0). Despite decreased β globin synthesis, however, the production of α globin continues and an excess of α globin therefore accumulates. These α chains form a highly insoluble tetramer (α_4) that precipitates in newly formed red blood cells. The α_4 precipitates damage red cell membranes, which leads to ineffective erythropoiesis and hemolytic anemia. Any measure that would increase the quantity of β-like globin molecules in the red blood cells of such patients would be of therapeutic benefit. One way to accomplish this goal would be

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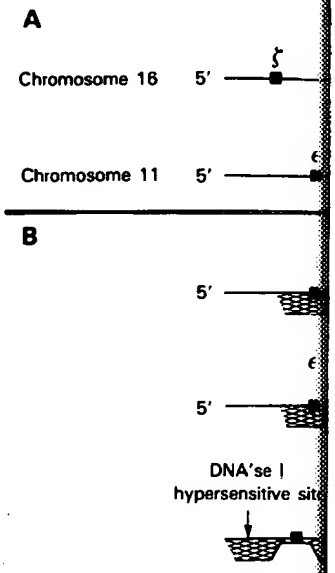

Figure 1 Panel A: Representatio
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Panel B: Chromatin structure and
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to reactivate expression of the γ globin genes, the structurally normal fetal counterparts of the β globin genes.

Functional Anatomy of the Human Beta-like Globin Genes

The human β-like globin genes lie clustered together on the short arm of chromosome 11. Five functional genes exist (see Figure 1*A*), and they are expressed consecutively during gestation. The first gene to be expressed is the embryonic (ε) gene, functional during the first 8–10 weeks of life. After the eighth week, ε gene function is replaced by that of the duplicated fetal ($^{G}\gamma$ and $^{A}\gamma$) globin genes. The fetal globin genes are active until late gestation,

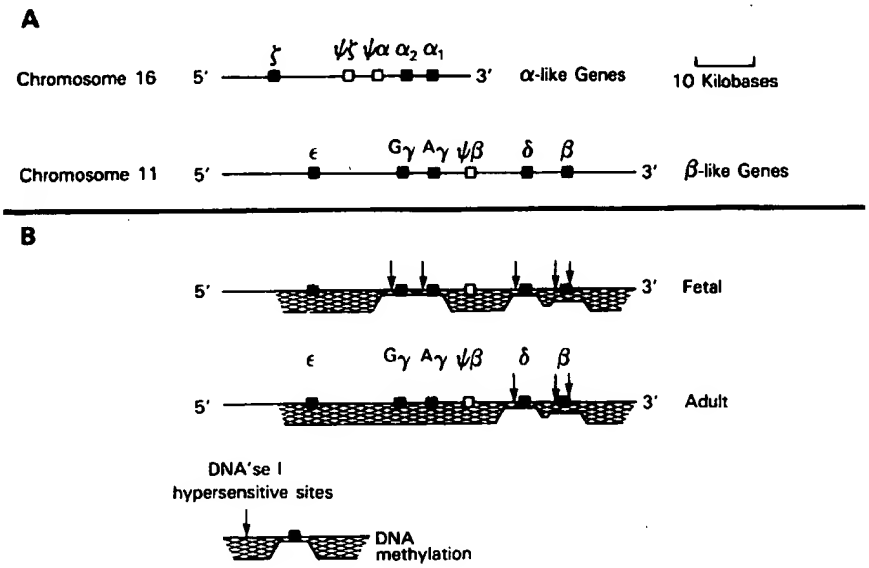

Figure 1 Panel A: Representations of the human α- and β-like globin gene clusters, lying on chromosomes 16 and 11 respectively. Functional genes are represented as black boxes, pseudogenes (nonfunctional genes homologous to the globin genes) are indicated by the open boxes, and flanking sequence DNA is represented as a line. Kilobase = 1000 base pairs.

In the α gene cluster, the zeta gene is expressed during the first 6–8 weeks of life, and the duplicated (α_2 and α_1) genes are expressed thereafter. For the α-like genes, only embryonic and adult-type genes exist; there is no counterpart to the γ (fetal) genes of the β-like cluster. Regulation of the β-like genes is discussed in detail in the text.

Panel B: Chromatin structure and DNA methylation near the β-like globin genes during the fetal and adult periods. DNAse I hypersensitive sites (nucleosome-free regions) are indicated by the bold arrows above the line. The extent of cytosine methylation is indicated by the hatched box below the line. A thick area of hatching indicates heavy cytosine methylation, and a thin region implies relative hypomethylation of cytosine residues. See text for details.

but become almost completely inactive after one year of life; the adult globin genes (δ and β) become active early in gestation, but are expressed at very low levels until just before the γ genes begin to be repressed. The δ gene is not capable of being expressed at high levels because it has a "defective" promoter (5). This defect is reflected by the small amount of hemoglobin A_2 ($\alpha_2\delta_2$) found in normal adult red cells.

Even though the fetal globin genes are fully active in fetal erythroid cells, these genes are expressed at low levels in adult red cells. Fetal globin gene expression in adults is manifest as a small number of red blood cells that contain HbF ($\alpha_2\gamma_2$). These "F-cells" comprise about 5% of circulating red blood cells, but only one sixth of the hemoglobin in these cells is HbF. This low-level activity implies that repression of the fetal globin genes is not complete, and that these genes may be "poised" for reactivation by various influences. Of note, some phenotypically normal adults have inherited genes that lead to the production of large quantities of HbF (hereditary persistence of fetal hemoglobin, or HPFH), a syndrome caused by a variety of molecular lesions (1).

Regulation and Modulation of HbF Synthesis in Red Blood Cell Precursors

Since only a small proportion of circulating red cells contain fetal hemoglobin, one would expect only a small number of erythroid stem cells to give rise to F cells. However, when adult human bone marrow cells are cultured in vitro with erythropoietin and fetal calf serum, the number of erythroid stem cells whose progeny produce HbF is greater than that predicted from in vivo observations (6). This implies that a developmental "program" of HbF biosynthesis is retained by many erythroid stem cells, and that this program can be reactivated under certain circumstances.

What determines whether the progeny of an erythroid stem cell will contain HbF? The answer is not yet clear, but it appears that this determination is made by a stochastic process early in the life history of the stem cell (7). The program for hemoglobin synthesis is not yet fixed in the earliest identifiable erythroid stem cells (the burst-forming unit–erythroid, or BFU-E), since some of the progeny of a single BFU-E stem cell may contain HbF, while others may not (8).

The quantity of erythroid stem cells that can produce F cells can be augmented by increasing the rate at which the stem cells divide. For example, when the bone marrow of humans is subjected to severe stress [e.g. recovery from bone marrow aplasia (9–11)], the erythroid stem cells divide more rapidly, and a larger number of their progeny produce HbF. The mechanism that causes this phenomenon is not known, but this observation suggests that in patients with β thalassemia HbF production could be increased by fo
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Molecular Correlates of Active Fetal Globin Gene Expression

The normal interactions of DNA with nuclear substances may be involved with the regulation of gene expression (12). DNA is normally associated with histones, highly basic nuclear proteins that organize DNA into nucleosomes. A nucleosome consists of an octomeric complex of histone proteins, around which are looped two turns of the DNA double helix. Nucleosomes can interact with other nuclear proteins to form the highly compacted structure known as chromatin. When DNA is packed in chromatin, it may be unable to interact with factors that regulate the rate of gene transcription (12). Near active genes, chromatin is organized more "loosely." In fact, near regulatory sequences located at the 5′ ends of genes, DNA often seems to be nucleosome-free. This may be a structural necessity for active gene expression, since regulatory substances and RNA polymerases could easily gain access to such DNA.

A summary of the chromosomal structures of the β-like globin genes during the fetal and adult periods is presented in Figure 1*B*. As expected, regions of nucleosome-free DNA (DNAse I "hypersensitive" sites) lie just upstream to the active γ globin genes of fetal erythroid cells (13, 14). Similar areas are also found next to the δ and β globin genes of fetal cells, which implies that these genes may be "poised" for later expression. This also suggests that, when both the fetal and adult genes are in an active conformation, the fetal genes may either outperform or repress the adult genes. Fetal-to-adult globin gene switching might therefore be due to γ gene repression rather than β gene activation. On the other hand, the β genes could conceivably be directly activated by a factor unique to adult erythroid cells, and the γ genes may become dormant secondarily.

A second functional correlate of active gene expression is the relative hypomethylation of cytosine residues near the 5′ end of active genes. Methylation of cytosine residues is the only significant postsynthetic modification of eukaryotic DNA; approximately 5% of cytosine residues are methylated, and most of these lie just 5′ to a guanine residue (15). The pattern of cytosine methylation near an individual gene is stably inherited and tissue specific, a finding that suggests cytosine methylation has some role in gene regulation (15, 16). Indeed, several studies revealed that heavily methylated genes are usually repressed. Conversely, DNA hypomethylation and active gene expression are also linked (16–18), but not all hypomethylated genes are transcribed. This implies that hypomethylation is necessary but not sufficient for active gene expression.

DNA hypomethylation and active globin gene expression are also closely associated. In embryonic erythroblasts, cytosine residues near the active embryonic globin genes are hypomethylated, while those near the fetal and adult globin genes are heavily methylated (17). In fetal erythroblasts, the fetal and adult globin genes are hypomethylated, but the embryonic gene is fully methylated (17, 18). Finally, in adult erythroblasts, the embryonic and fetal genes are repressed and heavily methylated. Additional studies by Busslinger et al (19) revealed that in vitro methylation of the 5′ end of the human γ globin gene extinguishes its expression in mouse L-cells; methylation of coding sequence DNA has no effect on expression. These basic observations suggested that hypomethylating DNA near the fetal globin genes might allow these genes to be reactivated in adult red blood cells. This was the hypothesis upon which the initial work with 5-azacytidine was founded.

STRATEGIES TO ACTIVATE FETAL GLOBIN GENE EXPRESSION

Altering DNA Methylation with 5-Azacytidine

5-Azacytidine is a cytotoxic, relatively S-phase-specific (20) cytidine analog that has been used experimentally for the treatment of acute leukemia since the early 1970s (21). This drug is incorporated into newly synthesized DNA (22–24), where it inhibits the normal enzymatic methylation of cytosine residues (23–25). The ability of this drug to inhibit DNA methylation has been correlated with its antileukemic effect in a mouse model (26).

DNA methylation is catalyzed by the enzyme DNA methyltransferase; when this enzyme encounters a 5-azacytidine residue in a newly synthesized strand of DNA, it probably becomes tightly bound to the 5-azacytidine (23–25). Binding of methyltransferase to 5-azacytidine inactivates this enzyme, so DNA synthesized in the presence of 5-azacytidine becomes hypomethylated within a few cell divisions. A variety of repressed genes can be activated in tissue culture cells grown in the presence of nontoxic amounts of 5-azacytidine (16), presumably because of the hypomethylating effect of this drug.

Armed with this information, DeSimone and Heller and their colleagues administered 5-azacytidine to phlebotomized baboons in the hope that this compound would reactivate the fetal globin genes (27). The stress of phlebotomy alone increased the *number* of F cells produced by these animals. However, when 5-azacytidine was administered, the *amount* of HbF per F cell also increased, a change that was accompanied by a reciprocal decline in HbA production. The augmentation of HbF synthesis was striking and reproducible, although the absolute increase in each animal was genetically predetermined.

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These observations prompted us to treat a small number of severely ill β-thalassemia patients to learn whether augmented HbF production would partially correct the unbalanced biosynthetic ratio of α to non-α globin, and thereby improve the effectiveness of erythropoiesis. Several patients with severe sickle cell anemia were also treated, since increased γ globin synthesis and decreased β^S globin synthesis could be of therapeutic benefit in these patients as well.

Eleven patients with severe β thalassemia or sickle cell anemia were treated at the Clinical Hematology Branch of the National Heart, Lung, and Blood Institute (28–30) and three additional sickle cell patients were treated independently at Johns Hopkins University (31, 32). All but one of these patients demonstrated a 4–7-fold increase in γ globin synthesis after administration of the drug. In several patients, we were also able to detect a small reciprocal decline in β globin synthesis similar to that seen in baboons (17, 29, 33). In the β thalassemia patients, the increase in γ globin synthesis outweighed the decline in β globin synthesis in each case, so the net effect was an improvement in the α-to-non-α globin biosynthetic ratio. This tendency toward normalization of the globin biosynthetic ratio improved the quality of erythropoiesis in these patients, since the absolute number of circulating reticulocytes increased with each course of treatment. However, augmentation of HbF synthesis was transient (lasting for only a few weeks after drug administration), so repeated courses of the drug were necessary to determine whether partial correction of the globin chain imbalance would yield therapeutic benefit.

The result of one such treatment course is depicted in Figure 2. The patient is a 29-year-old female of Mediterranean ancestry who is heterozygous for two different β globin gene mutations. Because of the nature of these mutations, the combined output of her β globin genes is about 5% of normal. 5-Azacytidine (2 mg/kg) was administered by subcutaneous injection three days each week for a total of six weeks. No transfusions were given during this interval. At the beginning of the treatment course, the patient's absolute reticulocyte count was approximately 5,000/mm^3, a level that reflected severe ineffective erythropoiesis and her recent transfusions. After several courses of the drug, however, the absolute reticulocyte count had increased to 48,600/mm^3, probably a sign of improvement in the quality of erythropoiesis. At the end of the treatment course, the absolute level of γ globin synthesis (with respect to α) had increased 6.2-fold, but a reciprocal 41% decrease in β globin mRNA production was also noted. The net improvement in the ratio of α to non-α globin was therefore approximately 4.6-fold.

Despite these changes, the patient's hemoglobin fell progressively during the treatment course. At the beginning of treatment, the hemoglobin was approximately 11.0 g/dl (reflecting recent transfusions); at the end, the

hemoglobin had stabilized in the range of 7 to 8 g/dl. The patient was symptomatic with this degree of anemia because of severe cardiac hemachromatosis, so transfusions were reinstituted. In this patient, and in another similarly treated, 5-azacytidine administration did not reduce or eliminate the need for transfusions (33).

The reasons for the lack of effectiveness of 5-azacytidine in these patients are probably many. First, the drug induces a reciprocal decline in β globin synthesis as γ globin synthesis increases. Second, repeated administration of the drug may cause subtle toxicity to the bone marrow. Although this patient's white cell count and platelet count were not altered, reduced numbers of erythroid progenitor cells have been observed in the bone marrow cells of some treated patients who had normal peripheral blood counts (30). Finally, patients of this kind are unable to tolerate even moderate anemia because of severe cardiac hemochromatosis (2).

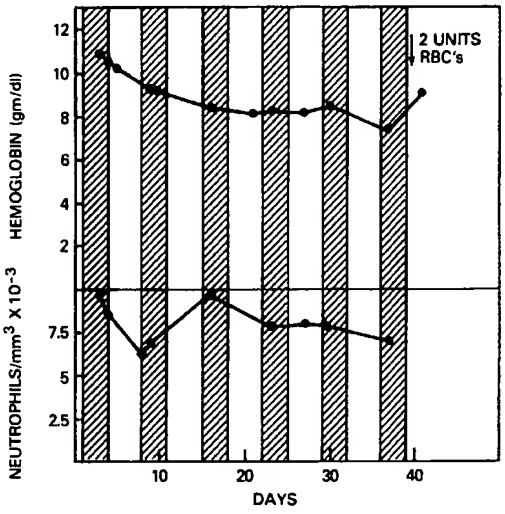

Figure 2 Hematologic data from a patient with homozygous β thalassemia who was treated with multiple courses of 5-azacytidine. The drug was administered as a single daily subcutaneous injection (2 mg/kg) on the days indicated by the hatched boxes. The neutrophil and platelet counts did not significantly change with treatment. Serial reticulocyte counts were not measured, but the patient's reticulocyte count before treatment was approximately 5,000/mm^3, as it had been previously with hemoglobin levels as low as 9 g/dl. On day 30 of the treatment course, the absolute reticulocyte count was 48,600/mm^3 and on day 41, it was 26,100/mm^3. The patient had no significant side effects during the treatment course, and was not transfused until day 40, when progressive exertional symptoms necessitated transfusion of two units of packed red blood cells.

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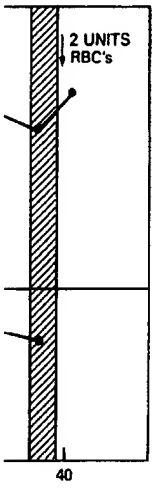

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These observations suggest that the thalassemic patients most likely to benefit from 5-azacytidine would be homozygous for β^0 mutations (i.e. no functional β mRNA is produced) so that no further decline in β globin synthesis would be possible. Furthermore, the patients most likely to benefit would have a genetic predisposition toward increased HbF production, since the maximal HbF synthesis with 5-azacytidine seems to be a function of the basal level (i.e. all patients experience a 4–7-fold increase in γ globin synthesis over baseline).

MECHANISM OF GAMMA GENE ACTIVATION WITH 5-AZACYTIDINE Since 5-azacytidine inhibits DNA methyltransferase, we expected the bone marrow cells of patients treated with a continuous infusion of the drug to exhibit "global" DNA hypomethylation at the end of treatment. Indeed, at least some cytosine residues became hypomethylated near the human γ and ε globin genes, the ζ (embryonic α) globin gene, the α2 [I] collagen gene, the insulin gene, and the C-myc proto-oncogene at the end of a continuous 7-day infusion. Other genes, like the α globin genes and the C-Ha-ras I proto-oncogene were hypomethylated in bone marrow cells before treatment, so methylation near these genes did not change. When the drug is administered by a daily injection, however, the areas of hypomethylation seem to be more limited, at least in the β globin gene cluster (31). This may be due to remethylation of transcriptionally "silent" regions of DNA during periods of the day when no 5-azacytidine is present.

Despite global hypomethylation of DNA, only the γ globin genes seem to be significantly activated in bone marrow cells by 5-azacytidine (28, 34). This observation has suggested to some that hypomethylation may not be mechanistically linked to γ gene activation. If this were true, the drug might then act by selectively "poisoning" the rapidly dividing late erythroid stem cells, causing the marrow to be repopulated by even younger stem cells whose progeny have an increased propensity to make HbF (7–11). Several lines of evidence support this hypothesis, at least in simian models.

When 5-azacytidine is administered to phlebotomized baboons (in doses higher than that used in our human studies), the number of late erythroid stem cells that can be cultured from the bone marrow decreases markedly (35). This indeed suggests that this drug may be selectively toxic to the rapidly dividing late erythroid progenitors, since 5-azacytidine is relatively S-phase specific (20). Other S-phase-specific cytotoxic agents, like Ara-C (36) or hydroxyurea (37), also cause an increase in HbF production in anemic primates. These drugs, which are not known to affect methylation directly, cause HbF responses similar to those seen with 5-azacytidine. At face value, these results suggest that 5-azacytidine may indeed act by causing selective toxicity to late erythroid progenitors, and that other cytotoxic agents will have identical effects in humans.

Studies of erythroid stem cells obtained from patients treated with 5-azacytidine indicate that the mechanism may be more complex. Some patients who respond to the drug do indeed have reduced numbers of late erythroid stem cells in the bone marrow after treatment. However, other responding patients demonstrate no change, and still others have *increased* numbers of late erythroid stem cells after treatment (30). Furthermore, when normal human bone marrow cells are incubated in the presence of nontoxic amounts of 5-azacytidine, the number of late erythroid stem cells that can be cultured from the marrow actually increases, and the amount of HbF synthesized by the progeny of these cells is also elevated (30). Finally, a *direct* effect of 5-azacytidine on the globin biosynthetic "program" of late erythroid precursor cells (e.g. orthochromatophilic erythroblasts) is suggested by the rapidity of γ globin induction in treated patients: increased numbers of HbF-containing reticulocytes appear in the peripheral blood within 1–2 days of starting the drug. These results suggest that 5-azacytidine may directly affect erythroid differentiation and the globin biosynthetic "program" of erythroid cells, possibly via its effect on DNA methylation.

Other experiments also suggest that the hypomethylating effect of 5-azacytidine is involved with γ gene activation. Ginder and colleagues (38) showed that expression of the embryonic β-like globin gene (ρ) of anemic adult chickens can be manipulated by a two-step process. First, the animal is treated with 5-azacytidine to hypomethylate genomic DNA, and then an "inducer" of cellular differentiation (sodium butyrate) is administered. This sequential combination of drugs activates the ρ gene in these animals. Neither drug, if given alone, affected ρ gene expression; furthermore, Ara-C administered with or without sodium butyrate had no effect. A similar experiment with the human globin genes corroborates these results. The human γ globin genes of an intact human chromosome 11 can be selectively activated in a cell line possessing "adult" erythroid properties. Gamma gene activation is accomplished by first treating the cells with 5-azacytidine, and then with the "inducing" agent hexamethylene bisacetamide (HMBA). Although 5-azacytidine caused widespread DNA hypomethylation, this alone was not sufficient to activate the γ genes in these cells (39). This experiment demonstrates that the γ globin genes can be activated by a process that does not involve manipulation of the normal pathway of erythroid differentiation.

Augmenting HBF Synthesis with Hydroxyurea

As noted above, Ara-C and hydroxyurea increase HbF synthesis in anemic primates. Hydroxyurea has also been used to stimulate HbF synthesis in patients with sickle cell anemia and β thalassemia (40; and G. Dover and A.

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W. Nienhuis, unpublished observations). Increased HbF production is evident in most sickle cell (but not β thalassemia) patients treated with the drug, but significant hematopoietic suppressi n can also occur. The degree of hematopoietic suppression correlated directly with the peak serum hydroxyurea concentration, and inversely with the rate of drug clearance. By adjusting the dose for each patient, hematopoietic toxicity could be avoided. From these studies, we learned that the most significant augmentation of HbF production occurred in the patients with the *least* hematopoietic toxicity.

There are several major differences between the effects of 5-azacytidine and hydroxyurea on HbF production in humans. Hydroxyurea's major effect is to increase the percentage of newly formed red cells that contain HbF, but it has little effect on the amount of HbF produced by each red cell. In contrast, 5-azacytidine increases both the number of F cells and the amount of HbF contained in each F cell. Finally, the rate at which HbF increases is faster with 5-azacytidine. Whether or not these clinical differences reflect differences in the mechanism of action of these drugs remains to be established.

Although hydroxyurea and 5-azacytidine are both capable of augmenting HbF production in humans, hydroxyurea has one major advantage as a potential therapeutic agent: unlike 5-azacytidine, hydroxyurea is thought to have essentially no carcinogenic risk for humans. 5-Azacytidine may have a potential to cause cancer in humans, but the relative risk is difficult to assess at this time (41, 42). To our knowledge, 5-azacytidine has not caused cancer in any patient treated with this drug, but the latent period is presumed to be quite long. This risk remains a major theoretical drawback of 5-azacytidine, and limits its use to severely ill β thalassemia or sickle cell anemia patients in an experimental setting. Further experimental work with 5-azacytidine has been deferred, pending the outcome of pilot studies with drugs like hydroxyurea and the results of chronic toxicity studies being performed in monkeys and rats.

Isolating Factors that Affect γ Gene Expression

Evidence is now accumulating that substances produced by genes not located in the β-like globin gene cluster are capable of affecting HbF production. For example, some patients with hereditary persistence of fetal hemoglobin have mutations that do not seem to lie in the β-like globin gene cluster (43, 44). In these patients, a γ gene repressor may fail to be produced (or a γ gene activator may not be shut off) at the appropriate time in gestation or red cell development. In addition, Papayannopoulou and Stamatoyannopoulos and colleagues recently demonstrated that a factor present in fetal sheep serum can reduce γ gene expression in cultured human

bone marrow cells (45, 46). Another factor that may influence globin gene expression has also been recently identified; Emerson & Felsenfeld (47) partially characterized a soluble nuclear protein that can bind near the chicken β globin gene promoter and induce an "active" chromosomal conformation in vitro.

These observations strongly suggest that regulatory factors encoded by another genetic locus may be responsible for the ordered switches in globin biosynthesis. The characterization and purification of these factors (and the genes that encode them) may provide therapeutic tools to modify γ gene activity in vivo. Finding a way to derepress (or activate) the γ globin genes with specific switching factors may provide a cure for β thalassemia and sickle cell anemia by creating an "artificial" hereditary persistence of fetal hemoglobin. With the power of molecular techniques at our side, this goal may be realized in the not too distant future.

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Introduction

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